Ultrasonic setup for testing hydrogels: preliminary experiments on collagen gels

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Abstract. The assessment of mechanical properties of highly hydrated natural materials remains a challenge because, in general, their mechanical evaluation implies invasive and finally destructive methods. Acoustic-based tests may represent the appropriate tools to investigate the mechanical properties of such materials, particularly collagen gels, whose acoustic properties are poorly understood. The objective of this work is to develop two experimental setups for the assessment of acoustic properties of such a hydrogels. In the first one, a typical pulse echo reflectometer was implemented. The acoustic parameters were measured at controlled temperature in an especially designed chamber. In the second one, the previous configuration was combined with a setup for compressive tests, allowing to interrogate simultaneously both the acoustic and mechanical properties of the sample under test. The frequency of the acoustic transducer was 10MHz. The acoustic and mechanical properties of collagen gels prepared according to different experimental conditions (pH and collagen concentration) were evaluated. The first set of experiment was useful to accomplish estimation of the speed of sound, attenuation and acoustic impedance. The second one allowed us to monitor the speed of sound during the evolution of the compression test. This approach could be a potential tool to study the changes in hydrogels mass density and bulk compressibility.

Introduction

In the last two decades tissue engineering approach has been applied to small caliber arteries grown in vitro [1]. Basically, cells are seeded into a matrix (scaffold) to build tissue equivalent in bioreactors. There are several materials used as scaffold, among others, collagen based gels are mostly used because their affinity with cells [2-3]. Non invasive evaluation of bulk mechanical properties of these highly hydrated collagen gels is a challenge due to its potential usefulness in bioreactors. Ultrasound (US) may represent the appropriate tool to interrogate the mechanical properties of such materials. Most of the contributions of the scientific community to the study of acoustic properties of collagen based gels come from the developing of gelatin phantoms (used in elastography and US medical equipment, see reference [4]). Despite of the fact that US has previously used in bioreactors [5] and neo-tissue grown in vitro [6], acoustical properties of the hydrated collagen gel matrices is poorly understood. The objectives of this work are, first, to acoustically characterize collagen gels with different mechanical properties. This is achieved using different collagen concentrations and pH (see subsection sample preparation). In subsection named experiment 1, a temperature-controlled set of US experiments is described.

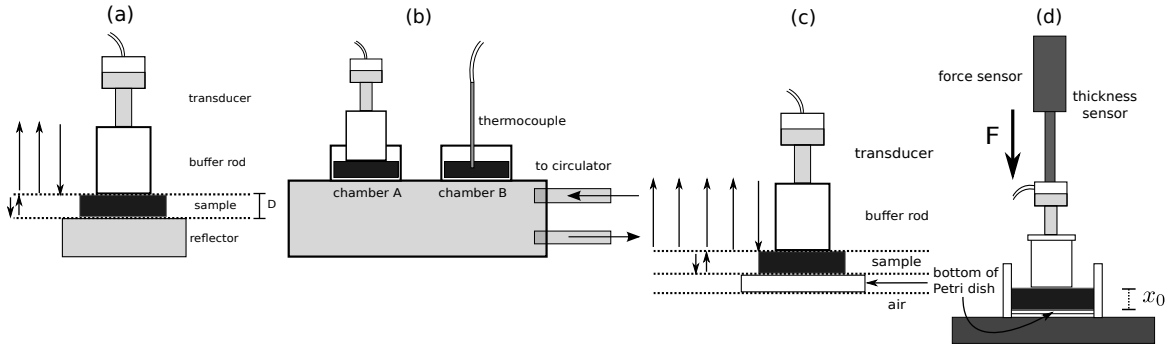

Fig. 1: (a-b) Experiment 1. Experimental setup to evaluate the acoustical parameters of gels and liquids at a controlled temperature. (c-d) Experiment 2. Setup to simultaneosly measure mechanical and acoustic properties of gels.

This was developed in order to obtain values of attenuation, acoustic impedance and speed of sound of collagen gels. The subsection named experiment 2 describes a setup which attempts to reach the second objective of this work: the study of correlations between the speed of sound and mechanical parameters of these gels.

Materials and Methods

Sample preparation. A solution of type-I collagen (4 g /L) and 0.02 M acetic acid was obtained from rat tail tendons (RTTs) according to a procedure previously developed [7]. Collagen gels were prepared at two different pH (7, 10) and with three different collagen concentrations (1.6, 2, 2.4 g/L, C1, C2, C3, respectively) according to a factorial plan. The acid collagen solution was mixed with 4 M sodium chloride (NaCl, 3.25%) and 1 M NaOH at different ratios in order to obtain collagen solutions at pH 10 and with the three different final concentrations of collagen. For all these collagen solutions the final concentration of NaCl was 130 mM. Collagen solutions at pH 7 were obtained by adding 1 M HEPES buffer solution. In this case, the final concentration of HEPES was 20 mM. The final volume was adjusted with deionized water. Collagen solutions were poured into Petri dishes (35 x 10 mm^2, Cell Culture Dishes, Corning Inc., Wilkes Barre, PA, USA) in a cold room at 4^{o}C and gels were allowed to set in there for half an hour. After that, samples were extracted from cold room and gelation process ended at room temperature (24^{o}C). NaCl solutions at different concentrations and gelatin samples were prepared in order to validate the experimental setup (data are not shown in this paper).

Experiment 1: Acoustic characterization of collagen gels. Disk-shaped samples (n = 3 for each concentration of collagen and pH) were extracted from the Petri dishes and placed carefully in chamber A (see Figs.1 a-b). A final diameter of 32mm and variable height ($<$ 7mm) was obtained. Samples were measured between intervals of 15 minutes in order let the temperature stabilize at 20^{o}C. US setup was based on reference [8]. Measurements were performed in a pulse-echo mode configuration. It consists in an US transducer attached to a buffer rod line (length 16 mm, diameter 15.6 mm) in contact with the sample and a total reflector, first and second layer (see Fig.1 (a)). The tuned transducer (Ultran LRD25-10, center frequency 10 MHz) was excited by an US generator (Ultran) in reflection mode. The signals returning from the sample were analyzed using a digital storage oscilloscope (HP 54616B 500MHz 2 GSample/sec). A total of 5000 points were acquired and sent to a computer by the RS232 serial port using especially developed software. Once signals were digitalized, three parameters were obtained: speed of sound (SOS), attenuation (α_s) and acoustic impedance (Z_s). SOS was obtained by

fixing the thickness of the sample at D=5mm (by gently pressing the US transducer until reach the 5mm thickness) and measuring the time difference of first echo (buffer rod / sample) and second echo (sample / reflector). The acoustic impedance was calculated by a reference medium (distilled water $Z_w = 1.4796\times10^6$ kgm^{-2}sec^{-1} at 20^{o}C [8]). This parameter was used also to calculate the attenuation. As a consequence, α_s and Z_s were referenced to distilled water values. The temperature controller was implemented by a circulator and an especially designed cell (see Fig.1 (b)). The cell has two chambers: the former to measure ultrasonic properties and the second one to measure the temperature by a thermocouple (OMEGA, connected to an i-Series 1/32 Programmable Temperature/Process Meters).

Experiment 2: US and mechanical properties. The objective of this experiment was to monitor the *SOS* during a mechanical compression test (see [9]). Disk-shaped samples were prepared as previously stated and tested in compression mode while US signal were recorded. The compressing plate consisted in the transducer + buffer rod directly applied to the sample. The cylindrical contact surface diameter was 15.6mm (see Fig.1 (c) and (d)) and it was attached to a 5 N load cell and an Instron Microtester (Instron 5848 Microtester, Instron Corporation, Norwood, MA, USA).
Acoustic measurements. Basically, the same US setup previously described was used, but in this case experiments were performed at room temperature (25^{o}C) and the sample container (Petri dish) was used instead the cell of Fig.1 (b). As a consequence there are more echoes in the US signal (see Fig.1 (c)). The sample thicknesses were obtained by the Instron, which has very precise increment measurements. The next procedure was applied. If two signals are taken, the measurements of two positions performed by the Instron are position 2 (p_2) (with its sample thickness $x_0 - \Delta x_2$ and time instant of the second echo t_2) and position 3 (p_3):

$$\begin{aligned} 2\cdot(x_0-\Delta x_2) &= SOS\cdot(t_2-t_0) \\ 2\cdot(x_0-\Delta x_3) &= SOS\cdot(t_3-t_0) \end{aligned} \tag{1}$$

where x_0 is the initial thickness, Δx_i is the increment that the Instron movement, t_0 is the time that arrive the first echo and t_2 is the one of the second echo that belong to the signal on p_2. The value t_3 is the time instant of the second echo of the signal on p_3. Therefore an estimate of the *SOS* can be obtained by taking the next equation (which is the least square minimization of Eq.1, using as many positions as possible, for instance: from 1 to 9):

$$SOS = \left(T'\cdot T\right)^{-1}\cdot T'\cdot X \tag{2}$$

where $\{\bullet\}'$ means transponse, T is the vector that contains the differences $t_{i+1} - t_i$ and X contains the differences $\Delta x_{i+1} - \Delta x_i$. It should be remarked that in this experiment only *SOS* measurements were performed.
Mechanical measurements. Samples were tested with a mechanical preconditioning of speed 0.02mm/sec and amplitude 0% to 2%. The speed of the test was also 0.02mm/sec. The stiffness curve was obtained by derivation of an interpolated from the compression data 5 order polynomial. Two parameters were defined (E_{R_I} and $E_{R_{II}}$) as the slopes of the strain-stress curves in two regions R_I and R_{II}, respectively. The strain level reached for R_I was $\varepsilon \approx 0.08$ and between $0.16 < \varepsilon < 0.33$ for R_{II}. The estimates of these two defined parameters were obtained by by taking the slope of an estimated linear polynomial from each particular region.

Statistical analysis. Three repetition of each sample of those enumerated in Table I were analyzed for the experiment 1 and another three for the experiment 2. Two way analysis of variance were carried out to evaluate the effect of the pH and the Collagen concentration. All

Table 1: Mean values (±standard deviation) of acoustic properties of collagen gels at 20^oC.

Sample (n=3)	$SOS[m/s]$	Attenuation (α_s/α_w)	$Z_s \times 10^6$ kgm^{-2}s^{-1}
C1 pH=7	1492.6 ±0.54	1.10±0.04	1.512±0.011
C1 pH=10	1490.8±0.50	1.15±0.03	1.510±0.007
C2 pH=7	1497.2±0.43	1.08±0.02	1.516±0.006
C2 pH=10	1495.3±0.37	1.13±0.01	1.514±0.002
C3 pH=7	1495.8±0.18	1.15±0.04	1.519±0.007
C3 pH=10	1494.2±0.21	1.18±0.06	1.518±0.016

the graphs were represented as mean ± standard deviation. The linear correlation coefficients between mechanical parameters and SOS were determined by using the Pearson correlation analysis. In all the mentioned tests, results were considered statistically significant for which $p - values < 0.05$.

Results and Discussion

It is remarkable that especial care was taken in preparing these kind hydrogels because of two important factors. The former is related to the air bubble content of the collagen gels: the faster the gelation process the more bubbles remains within the gel affecting its acoustical properties. The gelation process was delayed by making the solution at 4^oC. The second factor is the salt content. It is known [8] that SOS of solutions increase with NaCl content. Since the objective of this paper is not to measure the variation of salt content of the gels but their mechanical properties, all the samples were prepared with the same NaCl content (130mM). Regarding SOS in collagen gels for the sets of experiment 1, the results are presented in Table 1. The main objective of this set of experiment was to obtain mean values of the acoustic parameters of collagen gels. As far as we know, there are no studies regarding this topic. ANOVA tests showed significant differences ($p < 0.05$) between C1-C2 samples and between C1-C3 (the higher the concentration the higher the SOS), but the difference between C2-C3 was not significant. For the pH it was observed that the higher the pH the lower the SOS. Acoustic impedance was not significantly different for collagen concentration neither for pH factor. On the other hand, for the attenuation there was no significant difference with the pH factor but for the collagen concentration the attenuation of C3 showed the higher values ($p < 0.05$) as compared to C1 and C2.

Figure 2 shows a measurement of experiment 2 with the two defined regions. Figure 2 (a) shows the compression data. The US signals were acquired between 10 seconds, which is equivalent to the marked (x) strains. An US register has a length of 10μ sec (see Fig. 2 (b)), as a consequence is reasonable to suppose no displacement during its acquisition. It is also noted that R_I comprises three US registers; meanwhile R_{II} is between the fifth and the ninth. For each region a SOS is calculated using Eq. 2 and three measurements (SOS_{R_I} and $SOS_{R_{II}}$). It is defined other parameter by averaging all the registers (SOS_{MEAN}).

Figures 3 (a-c) show the results of mechanical parameters and SOS for the collagen content divided in the two defined regions. Statistically different values were found for the SOS_{R_I} and SOS_{MEAN} between the C1-C2 and C1-C3 but not for C2-C3: the higher the concentration the higher the SOS. On the contrary, this behavior was not observed in R_{II}, the results in this region were slightly in agreement with experiment 1 (see Fig.3 (d)).

Regarding the mechanical properties, in region I, strong differences were observed between parameters E_{R_I} and collagen concentration for C1-C2 and C1-C3 but not for C2-C3. This is

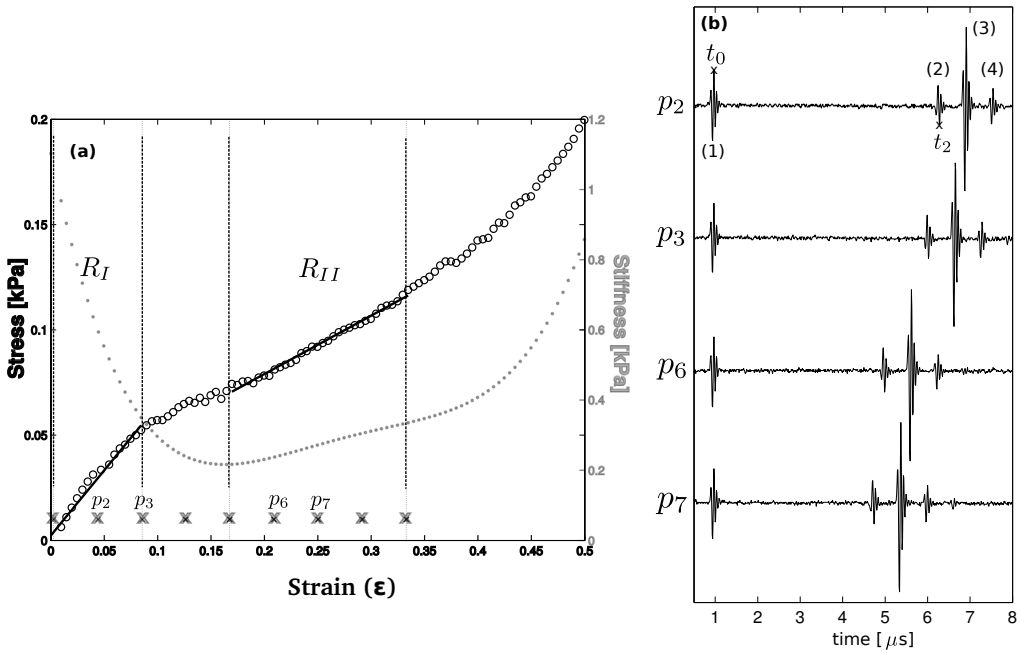

Fig. 2: Experiment 2. (a) Mechanical compression test on a collagen gel. Acoustic signals were acquired at the x marks. (b) Acoustic signals marked in (a).

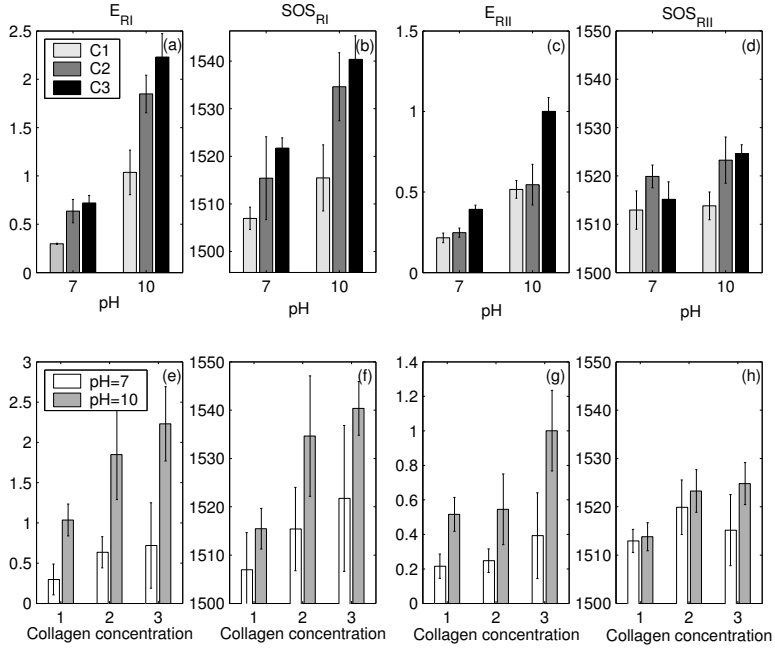

Fig. 3: Results on collagen gels. (a-d) Mechanical parameters and speed of sound, relations with collagen concentration. (e-h) Mechanical parameters and speed of sound, relations with pH.

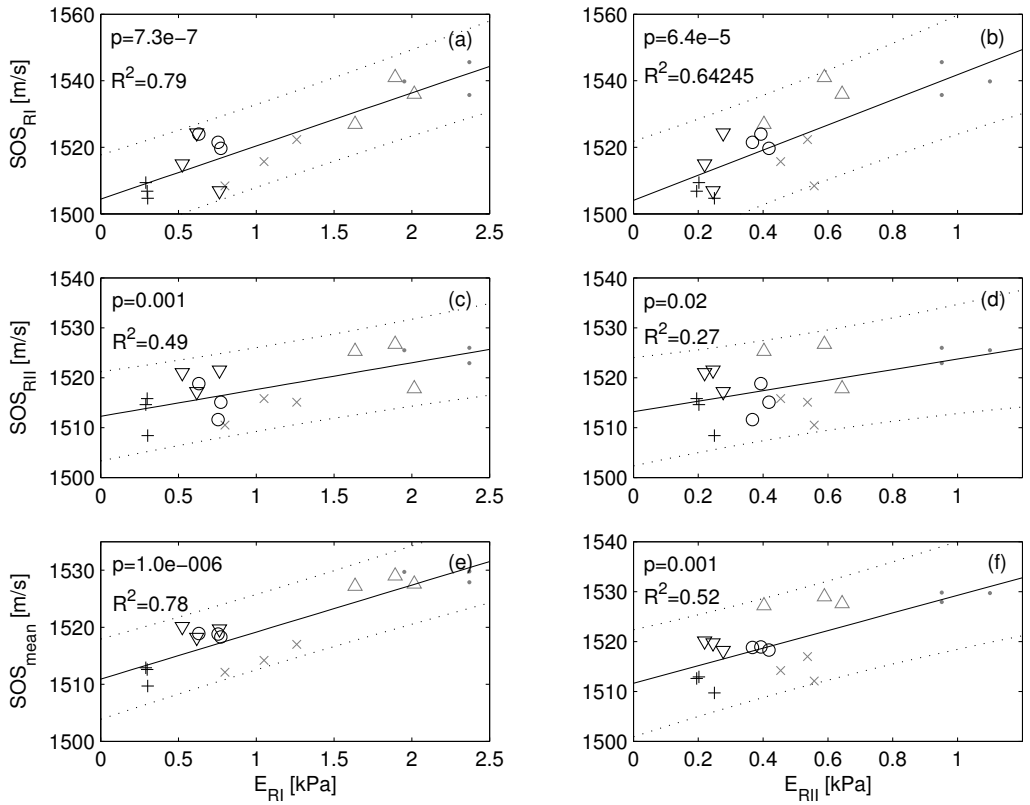

Fig. 4: Linear regression between mechanical parameters and speed of sound. Collagen concentrations 1.6/2.0/2.4 gr/L in + and x / triangle down and up / circle and dot, respectively. The pH 7 and 10 samples are marked in black and grey, respectively.

in agreement with the results found for SOS in this region. Figure 3 (c) shows an increasing in $E_{R_{II}}$ from concentration 1 to 3 and 2 to 3, but not for this value between 1 to 2. In Figs. 3 (e-h) the same data are shown but now plotted with the pH as x-variable. SOS in Region I and SOS_{MEAN} increased with the pH factor. Mechanical properties show similar behavior to those observed by Achilli et al [3]. In Region I and II very strong differences were observed between parameters E_{R_I}. An increasing in the pH from 7 to 10 corresponds to a higher value of E_{R_I}. This is not the case for the SOS in Region II, no significant differences were found.

Figure 4 shows a correlation analysis between SOS and mechanical parameters. There was a statistically significant linear correlation between SOS_{R_I} and E_{R_I} (r^2 =0.79 and p <0.01) (see Fig.4 (a)). This behavior is also observed with SOS_{MEAN} and E_{R_I} (r^2 =0.78 and p <0.01, Fig.4 (e)) and relatively between SOS_{R_I} and $E_{R_{II}}$ (r^2 =0.64 and p <0.01). Weak but statistically significant correlation was found between SOS_{MEAN} and $E_{R_{II}}$ (r^2 =0.52 and p <0.01). Regressions using $SOS_{R_{II}}$ parameter were always poor predictors of E_{R_I} (see Fig.4(c and d)). Regarding Fig.4, it is remarked that the material under test was always in different conditions (collagen content and pH), therefore results must be taken carefully. As a general behaviour, it could be pointed out that the defined mechanical parameters are well predicted by either SOS_{R_I} and SOS_{MEAN}. In order to compare the results of both set of experiments it must be remarked that in experiment 1 the disk-shaped samples were extracted from the dishes and gently pressed until reach the 5mm thickness. This protocol generates two undesired effects: lose of water and a non-controlled strain (which is probably greater than 0.16, $\varepsilon > 0.16$). If this interpretation is correct data are in relative good agreement (see Fig. 3 (d) and (h)). As it is shown in Figs. (2-4), liquid content has a strong influence in both; mechanical and acoustical

properties. Apparently, in Region I the obtained parameters are due to the equilibrium between the network (matrix) and the liquid. In Region II, liquid is flowing outside the network and consequently the material is modified. It is important to remark that the same behavior is observed in the *SOS*.

Conclusion

In this work two ultrasound experiments were developed in order to interrogate the acoustical properties of collagen gels at 10MHz and their correlation to mechanical properties. The values obtained for the attenuation are very similar to those observed in deionized water. Higher values were found for the acoustic impedance instead. It must be noted that these data were obtained by applying strain greater than 0.16. As it was observed in experiment 2, it seems that speed of sound of collagen gels is a parameter sensitive to this phenomenon.
Further development and study is necessary to understand the mechanical behavior in the two regions, which apparently is also observed in the speed of sound. It is also interesting to notice that mechanical properties and speed of sound show high correlations, which make this approach very attractive in real time monitoring of bioreactors.

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