Dynamic Depth-dependent Osmotic Swelling and Solute Diffusion in Articular Cartilage Monitored using Real-time Ultrasound

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ABSTRACT

The objective of this study was to investigate the feasibility of ultrasonic monitoring for the transient depth-dependent osmotic swelling and solute diffusion in normal and degenerated articular cartilage (AC) tissues. Full-thickness AC specimens were collected from fresh bovine patellae. The AC specimens were continuously monitored using a focused beam of 50 MHz ultrasound during sequential changes of the bathing solution from 0.15 M to 2 M saline, 0.15 M saline, 1 mg/ml trypsin solution, 0.15 M saline, 2 M saline and back to 0.15 M saline. The transient displacements of ultrasound echoes from the AC tissues at different depths were used to represent the tissue deformation and the NaCl diffusion. The trypsin solution was used selectively to digest the proteoglycans in AC. It was demonstrated that high frequency ultrasound was feasible for monitoring the transient osmotic swelling, solute transport and progressive degeneration of AC in real time. Preliminary results showed that the normal bovine patellar AC shrank during the first several minutes and then recovered to its original state in approximately 1 h when the solution was changed from 0.15 M to 2 M saline. Degenerated AC showed neither shrinking nor recovering during the same process. In addition, a dehydrated-hydrated AC specimen showed much stronger shrinkage and it resumed to the original state when the solution was changed from 2 M back to 0.15 M saline. The diffusion of NaCl and the digestion process of proteoglycans induced by trypsin were also successfully monitored in real time. (Email: rczheng@polyu.edu.hk)

Keywords: ultrasound, articular cartilage, osteoarthritis, osmotic swelling, solute diffusion

INTRODUCTION

The articular cartilage (AC) is the thin layer of connective tissue that covers the articulating bony ends in diarthrodial joints. In the last few decades, AC has attracted a tremendous amount of interest from researchers because of its important functions and unique structural characteristics, such as multiphasic compositions and multilayered structures, special diffusion-based nutrition transportation and frequent degeneration with aging (Mow et al. 1991). The extracellular matrix of AC contains negatively charged proteoglycans embedded in a collagenous network (Fig. 1a and 1b). In the biological condition, there is a balance between collagen tension and swelling pressure, contributed by the negative charges (Maroudas 1976a; Lai et al. 1991). This balance plays an important role in the unique biomechanical behavior of AC. Factors such as proteoglycan concentration, water volume fraction and the intrinsic material properties of the AC solid matrix govern the inhomogeneous swelling strain distribution *in situ* (Maroudas 1976a; Lai et al. 1991; Narmovena et al. 1999). These factors also affect the diffusivity of various solutes through the surface of the AC and within the extracellular matrix. These solutes are essential for transporting nutrients, metabolic waste products and molecules for cell signaling between the synovial fluid and the chondrocytes (Maroudas 1976b; Quinn et al. 2001). It has been suggested that the quantification of the swelling effects in AC can be used to characterize the degenerative changes associated with osteoarthritis (Maroudas 1976a; Narmovena et al. 1999). Previous investigations have also demonstrated that the degeneration of AC may increase the diffusivity of solutes (Lotke and Granda 1972; Burstein et al. 1993; Xia et al. 1995; Torzilli et al. 1997).

The swelling properties of AC can be characterized mechanically in tension (Grodzinsky et al. 1981; Guilak et al. 1994) and in compression (Eisenberg and Grodzinsky 1987), or electromechanically in compression (Berkenblit et al. 1994). Depth-dependent swelling behaviors were previously studied destructively by cutting a full-thickness slice of AC into layers and testing the layers individually (Guilak et al. 1994). Recently, optical methods have been developed to characterize the equilibrium swelling behavior of full thickness AC sections (Narmoneva et al. 1999, 2001, 2002; Setton et al. 1998; Flahiff et al. 2002). To derive the local strains induced by swelling, optical microscopic images of the cut surfaces of full-thickness AC were recorded under different concentrations of bathing saline solution and compared. As the AC at the cut surface of the specimens disintegrated, these optical methods were not feasible for *in-vivo* applications or for investigating the *in-situ* transient behaviors of swelling and solute transport.

Previous investigations of the transport of solutes in AC have employed different methods, including radiolabel (Torzilli et al. 1997; Maroudas 1976b; Schneiderman et al. 1995), magnetic resonance (Burstein et al. 1993; Xia et al. 1995), fluorescence tracer methods (Quinn et al. 2001) and scanning electrochemical microscopy (Gonsalves et al. 2000). Among them, magnetic resonance imaging (MRI) methods show great potential for use in nondestructive *in-situ* measurements of depth-dependent solute diffusion. However, they are expensive and most MRI systems are limited to a relatively low spatiotemporal resolution. An easy and nondestructive approach for measuring the *in-situ* transient transport of solutes in AC is still lacking.

The ultrasonic characterization of AC has been the subject of many recent investigations, due to its nondestructive manner and penetration ability. These investigations have included studies on the assessment of progressive changes in the AC with experimental osteoarthritis (Saied et al. 1997), the monitoring of the ultrasound parameters of AC during enzyme digestion (Nieminen et al. 2002) and the measurement of the inhomogeneous mechanical properties of AC during compression (Zheng et al. 2001, 2002, 2003a; Fortin et al. 2003) (Refer to Zheng et al. (2003) for a full list of relevant references). Local strains of AC tissues under compression can be measured or imaged using ultrasound so as to characterize AC in a nondestructive way. However, the collection of scattered high-frequency ultrasound signals from AC tissues during compression still remains a challenge, particularly when the AC specimen is small, such as a mouse joint (Saied et al. 1997). The osmotic swelling of partially dehydrated normal AC as a whole layer has previously been probed using ultrasound (Tepic et al. 1983). However, due to technical limitations, no measurement was achieved for the depth-dependent swelling behavior. In addition, the potentials of ultrasound for measuring the transport of solutes and swelling in AC induced by changing the concentration of bathing solution has not been reported. We have developed an easy approach using 50 MHz ultrasound for AC research. Our aim was to use this approach to monitor and compare transient depth-dependent swelling behavior and solute transport between normal and degenerated AC.

MATERIALS AND METHODS

AC Specimens

Cylindrical AC specimens with a bone layer approximately 3 mm thick were prepared from fresh mature bovine patellae using a metal punch with a diameter of 6.35 mm (Zheng et al. 2001, 2002). One AC disk was selected from each patella for the ultrasound-swelling test. The prepared specimens were stored at –20°C until the ultrasound measurement. The results of six specimens were reported in this paper and used to address different aspects of this technique. A trypsin solution was used to digest the proteoglycan to induce AC degeneration (Zheng et al. 2001). One of the specimens had been prepared and dehydrated eight months before the test was conducted for this study. During the test, it was first thoroughly hydrated in a 0.15 M saline solution for more than 6 h.

Ultrasound System

A 50 MHz ultrasound system was recently developed to monitor and compare the normal and degenerated AC specimens in terms of the transient depth-dependent swelling behavior and the transport of solutes induced by changing the concentration of bathing saline. Figure 2 shows the schematic diagram of the ultrasound swelling measurement system used in this study. The system included a 50 MHz focused ultrasound transducer (focal length is 12.3 mm and -6 dB focal zone is approximately 1 mm in length and 0.1 mm in diatemeter; Panametrics, Waltham, MA, USA), an ultrasound pulser/receiver (model 5601A; Panametrics, Waltham, MA, USA), an A/D converter card with a

sampling rate of 500 MHz (model CompuScope 8500PCI; Gage, Canada), and software for data collection and signal processing (Zheng et al. 2003). The resolution for displacement measurement was approximately 0.4 μm in AC using a cross-correlation algorithm and an average sound speed of 1666 m/s in AC (Zheng et al. 2002). This displacement resolution was sufficient to allow detection of a 0.4% deformation for a 0.1 mm thick layer of AC.

Ultrasound Measurements

AC specimens were first taken from a -20°C freezer, placed rigidly on the bottom of the container and then submerged in a 0.15 M saline solution for thawing (Fig. 2). The sides of the AC specimen were gently sealed using rubber gels to ensure that the fluid was transported only through the surface of the AC. The ultrasound transducer was moved to a position over the central portion of the AC specimen, with the focal zone of its beam located inside the AC layer. The AC specimen was tested in room temperature (19.5±1°C) for all of the following procedures. After the AC specimen had been thawed for 3 h, the 0.15 M saline was replaced with 2 M saline within 30 s, and the AC was monitored with ultrasound for 1 to 2 h. The echo signals that reflected from the surface of the AC and from the AC/bone interface and scattered inside the AC layer were continuously recorded with a sampling period of approximately 1 s. Figure 1c shows a frame of typical ultrasound radio-frequency (RF) signals collected from an AC specimen (#1). The ultrasound signals were also displayed in an M-mode image to represent transient information. This means that the echoes at different measurement times were

drawn line-by-line to form an image, with the grey levels indicating the amplitude of the ultrasound signals (Fig. 1d). Each horizontal trace in the M-mode image indicated the transient displacement of the AC tissues at different depths, induced by the change in saline concentration. The bathing solution was further changed from 2 M saline to 0.15 M saline, a 1 mg/ml trypsin solution, 0.15 M saline, 2 M saline and 0.15 M saline for different periods of time (Fig. 3). The ultrasound signals were recorded and displayed with M-mode images during the whole procedure except during the replacement of solutions, which lasted for approximately 30 s. To confirm that the different swelling behaviors of AC tissues before and after the digestion of trypsin were not due to other changes in the specimen during the long period of the experiment, another AC specimen was tested following the whole procedure described above, but with the trypsin solution replaced by a 0.15 M saline solution during the “digestion” period. Additional experiments were also conducted by changing the concentration of the saline solution from 0.15 M to 0.015 M and then back to 0.15 M. A repeatability test was conducted on a specimen (#6) with a procedure of changing the concentration of the saline in a sequence of 0.15, 2, 0.15, 2 and 0.15 M. For each concentration of saline, the ultrasound echoes were recorded for approximately 1 h.

RESULTS

Figures 3a to f show the M-mode representation of the ultrasound signals collected from AC specimen #2 during different processes. After the saline was changed

from 0.15 to 2 M, the AC surface of the normal specimen first gradually moved downwards and then recovered almost to its original position after approximately 1 h (Fig. 3a). The same phenomenon was observed in the four fresh specimens that we measured. The echo reflecting from the AC/bone interface gradually moved upwards, indicating that a shorter time was required for the ultrasound to propagate through the AC. It means that a change had occurred in the speed of the ultrasound in the AC during this process and that this change was the result of the penetration of the 2 M of saline into the AC tissues (i.e., a diffusion of NaCl) from the superficial to the deeper zone. Since the bone was fixed to the bottom of the container, the movement of the AC surface could be used to represent the change of AC thickness. After the saline was changed from 0.15 to 2 M, the thickness of the AC was first reduced and then almost resumed to its original value. At the beginning of the process, as shown in Fig. 3a, the diffusion of NaCl could be traced by tracking the disturbance of the ultrasound signals, as indicated by the dotted line. Using the change in the ultrasound speed of the AC and the disturbance in the ultrasound signals, the diffusivity of NaCl at different depths could be estimated. The displacements of AC tissues at different depths could be estimated by tracking the horizontal traces. The transient change in the speed of the AC ultrasound at different depths might also contribute to the shifts in the ultrasound signals represented in a time scale. As shown in Fig. 3b, the displacement of the AC was very small when the saline solution was changed from 2 M back to 0.15 M, although the upward movement of the echo that was reflected from the AC/bone interface suggested that the 0.15 M saline solution gradually penetrated into the tissue. This phenomenon demonstrated that the swelling processes

induced by changing the concentration from 0.15 M to 2 M and back to 0.15 M was not reversible. Similar displacements of the AC surface and changes in the ultrasound speed of the AC were observed in the tests by changing the saline concentration between 0.015 M and 0.15 M. However, the values were much smaller in comparison with the cases of 0.15 M to 2 M.

Figure 3c shows the ultrasound signals collected during the process of trypsin digestion, which lasted for approximately 6 h. The digestion was clearly observed from the additional echoes generated at the interface between the digested and undigested AC tissues. The shift of these additional echoes formed the inclined streak in Fig. 3c. The penetration rate of the trypsin solution or the digestion rate could then be estimated with this inclined trace. The echo from the bone/AC interface shifted slightly, indicating a corresponding slight change in the speed of the ultrasound after the AC had been digested by trypsin. After the digestion with trypsin, the AC specimen was submerged in a 0.15 M of saline solution for 3 h. Interestingly, the digestion process continued during this period, as indicated by the inclined trace in Fig. 3d. Better to represent the additional echoes generated at the interface of the digested and undigested AC tissues, Fig. 4 shows three typical radio-frequency ultrasound echo trains that were collected at the time positions indicated by the three vertical dashed lines A, B and C in Figs. 3b, c and d. Indicated by the dashed circles, additional echoes were generated at different depths of AC as the trypsin digestion penetrated into the deep region as time went on. The trypsin digestion for proteoglycan appears not to affect the scatterers in AC tissues, as the patterns of scattering signals in the digested region did not change after the digestion front

penetrated into deeper region. However, the amplitudes of the scattering signals tended to increase as the digestion time increased ,as a result of the fact that more ultrasound energy might be transmitted into the AC specimen. This observation agreed with the previous finding that less energy was reflected from the AC surface after trypsin digestion (Nieminen et al. 2002).

In the process of changing the saline from 0.15 to 2 M and back to 0.15 M, the surface of this digested AC no longer shifted, although the shift in the echo from the AC/bone interface indicated that the 2 M saline solution had penetrated (Fig. 3e). Again, from each trace of the echoes, the diffusivity of NaCl in 2 M of saline could be estimated. When the concentration of the saline was changed from 2 M back to 0.15 M, the movement of the echo from the AC/bone could be observed, but not that from the surface of the AC (Fig. 3f). Since no swelling was observed in the digested AC, the shifts in the echoes were solely due to the change in the speed of the ultrasound induced by the diffusion of NaCl. Thus, swelling-induced transient strains at different depths of AC before trypsin digestion could be obtained by correcting the echo traces in Fig. 3a using those in Fig. 3e.

For specimen #3, when the trypsin solution was replaced by 0.15 M of saline during the processes, as shown in Fig. 3, the swelling-induced movement of the AC surface was observed at the beginning of the test (Fig. 5a), as well as after approximately 10 h into the experiment (Fig. 5b). It further supported that the changes in the swelling behavior of AC shown in Fig. 3 were caused by the removal of the proteoglycan that had been induced by the trypsin digestion and not by other effects such as the duration of the

experiment and the thawing process at the beginning of the test. In addition, the transient diffusion of NaCl in the 2 M saline was also identified, according to the disturbance in the ultrasound signals and shifts in the echoes from the AC/bone interfaces (Figs 5a and b).

In another test, the specimen (#4) was stored at -20°C after it had been digested for 6 h and submerged in 0.15 M of saline for 3 h. One day later, a swelling test for this digested specimen was conducted again. After the specimen had been thawed in 0.15 M of saline for 3 h, the saline was replaced with 2 M and then back to 0.15 M. No surface displacement of this digested specimen was observed during the process (Figs 5c and d). It further supported again that the swelling behavior was altered by the digestion process and not by other effects such as possible incomplete thawing at the beginning of the test.

The swelling-induced displacements of AC tissues appeared to be much larger for the dehydrated/hydrated AC specimen #5 (Figs. 5e and 5f). In addition, the recovering pattern of the displacements observed in the fresh specimens could not be observed in the dehydrated/hydrated AC specimen. When the concentration of saline was changed from 2 M back to 0.15 M, a stronger reversing process of the tissue displacements was observed, which had not occurred in the fresh AC specimens.

The repeatability test demonstrated that the measurement of the transient shifts of the echoes was repeatable. As shown in Fig. 6, the patterns of the transient echo shifts were very similar for the two consecutive tests. When the saline was changed from 0.15 to 2 M, the transient echo shifts measured at the two tests were almost overlapped with

each other for both echoes from the AC surface and the AC/bone interface. Similar repeatability was obtained for the shifts of the scattering echoes from tissues at different depths of AC. The shift of the echo from the AC surface represented the thickness change of AC tissues and the shift of the echo from the AC/bone interface represented the change of the ultrasound speed in the AC tissues. Hence, the measurement of the transient displacements of the tissues and the sound speed would also be repeatable.

DISCUSSION

We demonstrated that a high frequency focused ultrasound beam could be used to monitor various transient processes of articular cartilage (AC) at different depths, including the swelling induced by changing the concentration of bathing saline, the diffusion of NaCl and the digestion of proteoglycan induced by trypsin solution. Our results demonstrated that the transient swelling behavior of AC altered dramatically when the proteoglycan was digested with trypsin enzyme, simulating the natural degeneration process. Because of its nondestructive feature and high sensitivity, this ultrasonic approach may potentially be used to diagnose cartilage degeneration in its early stages *in vivo* together with an arthroscopy. The maximum deformation of normal AC induced by changing the saline concentration from 0.15 M to 2 M occurred during the first several minutes. Thus, with proper miniaturization of the instrument, it would be clinically practicable to detect the cartilage using the approach introduced in this paper. In comparison with the promising MRI diagnosis of AC *in vivo* (Burstein et al. 1993; Xia et al. 1995), the present ultrasonic approach may allow real-time monitoring of transient depth-dependent swelling and solute transport. In addition, ultrasound measurements of

swelling and solute transport may be more accessible and much cheaper in comparison with the current MRI.

The transient depth-dependent displacements of AC tissues during swelling may provide comprehensive information on the biomechanical behaviors of AC, which is currently unavailable. The various biomechanical models of AC found in the literature can be used to extract the intrinsic material properties of AC and new biomechanical models of AC might be devised to explain new findings. We observed that AC tissues first appeared to shrink but then expanded after approximately 10 min, almost returning to their original state after approximately 40 to 100 min when the concentration of the bathing saline was changed from 0.15 to 2 M. This self-recovering phenomenon was repeated for all of the measured normal specimens. The repeatability was also demonstrated by the results of a specimen tested twice with a 10 h interval, during which the specimen was submerged in saline solutions with different concentrations. In addition, one specimen was monitored for 4 h after the saline was changed from 0.15 to 2 M, and no further shift in the surface of the AC was observed after the phase of resumption.

The new findings about the transient swelling behavior of AC differ from the common understandings, including equilibrium results measured using optical methods where the free swelling of the cut surfaces of the AC were monitored (Berkenblit et al. 1994; Setton et al. 1998; Narmoneva 2001, 2002; Flahiff et al. 2002), transient results measured in tension for AC stripes (Grodzinsky et al. 1981) and the current models for describing the swelling behavior of AC (Lai et al. 1991). Since only the central region of

approximately 0.1 mm in diameter was monitored for the full-thickness AC specimen with 6.3 mm in diameter, the reported results were well representative for an *in-situ* condition. For the specimen that was previously dehydrated, the shrinkage induced by changing the saline concentration from 0.15 to 2 M was much stronger and the recovery weaker. It appears that some components of the AC or some linkages between different components were damaged in the dehydrated specimen, resulting in a change in the transient swelling behavior. However, the results of the limited number of specimens would not be enough to let us make a clear explanation for the observed phenomena. More specimens from different regions of patella as well s from other joint parts will be tested in the future. In addition, in order better to understand this recovering phenomenon, we plan to remove different components of AC sequentially by using the enzymatic digestion of the AC specimen with different enzymes, including collagenase for removing collagen fibers and trypsin for removing proteoglycan. We believe that the comprehensive transient and depth-dependent information provided by this ultrasound-swelling approach, together with the ultrasound-compression approach (Zheng and Mak 1996; Zheng et al. 2001, 2002, 2003a; Fortin et al. 2003), may give more experimental support for the current biomechanical models of AC (Lai et al. 1991; Mow et al. 1980, Mak 1986) and stimulate more advanced modeling.

The precise and reliable real-time monitoring of the digestion process of enzyme provided a unique tool for the preparation of AC specimens to model degenerations. Similar studies have been reported using 30 MHz ultrasound (Nieminen et al. 2002). Since the raw radio-frequency ultrasound signals with higher frequency were used in this

study, M-mode images with a higher resolution showed clearer traces of the penetration of trypsin. Previous studies demonstrated that the trypsin penetration front measured with ultrasound correlated with that measured with histology (Zheng et al. 2001; Nieminen et al. 2002; Qin et al. 2002). The trypsin digestion of proteoglycan in AC could reduce the Young's modulus of the tissues (Zheng et al. 2001) and subsequently introduce the discontinuity of acoustic impedance in the interface of the digested and undigested regions. This interface moved towards the deeper region as the digestion progressed. The acoustic impedance also depends on the tissue density. However, a previous study demonstrated that the AC density was not affected by removing the proteoglycans using enzymes (Gu et al. 1999). We are now able to prepare a partially-degenerated AC specimen by replacing the trypsin solution with a proper enzyme inhibitor when the digestion front reaches a required depth. As both the enzyme digesting and inhibiting process can be real-time monitored, it is possible to know immediately whether the specimen is properly prepared. Currently, preparation of such AC models is normally verified with histology, which is a destructive method and can only be performed after the digestion. As the trypsin digestion depth can be precisely controlled, the functional contributions of proteoglycans at different AC depths can be easily studied using these models. Therefore, in order to understand how different zones of AC contribute to swelling and solute transport, we also plan to conduct swelling tests for specimens with precisely controlled different digestion depths.

In the present experiment, the ultrasound speed in AC tissue was found to have become larger in the saline solution with the higher concentration. When the

concentration of the saline was changed, the diffusion of NaCl could be reliably monitored by observing the disturbance in the ultrasound signals as well as the change in the speed of the ultrasound, which was indicated by a shift in the echoes from the AC/bone interface. Our results demonstrated that the diffusivity of NaCl in normal and degenerated AC was not dramatically different. This agreed with the previous results that the AC diffusivity of molecules with a small molecular weight might not be altered by the removal of proteoglycan (Torzilli et al. 1997). This new ultrasound approach may provide a nondestructive way to measure the transport of solutes in AC at different depths for molecules with different molecular weights, so as better to understand the transport of nutrients and cell signaling elements in AC (refer to Quinn et al. (2001) for a summary of molecules that have been studied using different approaches). Previous studies reported that the ultrasound speed of AC was depth-dependent (Agemura et al. 1991; Patil et al. 2003). There was a difference of approximately 10% from the superficial zone to the deep zone, according to our recent results. This issue has to be taken into account when extracting quantitative depth-dependent information from the echo traces in the M-mode images. In addition, the shift in the ultrasound echo from AC surface could also be caused by a change in the sound speed in water induced by the temperature change. All of the echoes from the AC would be altered equally by this factor and shifts in echoes could be corrected by measuring the ultrasound speed of saline at different temperatures. In the experiments presented in this paper, the room temperature was controlled and measured as 19.5 ± 1ºC. Our recent study on the temperature-dependency of the ultrasound speed in AC based on 20 patellar specimens showed that the ultrasound speed

in full-thickness AC and 0.15 M saline increased by 1.9% and 1.8%, respectively, when the temperature was changed from 18.5ºC to 20.5ºC. The changes of ultrasound speeds in AC and saline could cause some uncertainties to the measurement results. However, these uncertainties were much smaller in comparison with the measured displacement of the interested echoes. The potential artifacts caused by the temperature change can be further reduced by using a temperature controlled chamber in the future studies.

In summary, the ultrasonic technique introduced in this paper provided a new approach for studying the biomechanical and biophysical problems associated with AC, including swelling, solute transport and progressive degeneration. We observed, using this approach, a self-recovering phenomenon of the normal AC after the concentration of its bathing solution was changed. The mechanisms behind this interesting phenomenon are still not clear and more experimental and theoretical investigations need to be followed. This ultrasonic approach may have wide applications in the study of various dynamic processes of AC *in situ* at different depths under biochemical, bioelectrochemical, biophysical, biomechanical and other types of stimulations, in animal models and patients as well as in cultured or tissue-engineered AC. Similar approaches can also be used for characterizing other tissues including the intervertebral disc, tendon etc. The reported results of this new approach are still preliminary and advanced signal and image processing algorithms are required to derive more quantitative parameters. One of the aims is to separate the effects of the sound speed changes and the swelling induced displacements at different depths in AC after the saline concentration is changed. In spite of these challenges, this ultrasonic approach is noncontact and nondestructive,

has a high resolution and requires little or no preparation of the specimens. As a result, it could be used to investigate the biomechanical properties and solute transport of the AC of small animals like rats and mice, which is currently still very challenging. By miniaturizing the probe and designing a proper mechanism for altering the concentration of NaCl and other solutes locally on the AC surface, this technique can potentially be used for the *in-vivo* detection of early signs of osteoarthritis, together with arthroscopy.

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FIGURE CAPTIONS

Fig. 1. (a) Schematic representation of the AC structure with the collagen fibers arranged differently at different zones (Mow et al. (1991)); (b) Histology of a typical AC specimen with the red color (darkness in the gray image) indicating the concentration of proteoglycan stained with Safrannin-O; (c) A-mode ultrasound signal obtained from a typical specimen (#1); (d) M-mode representation of the ultrasound signals collected during swelling, with the grey level indicating the signal amplitude shown in (c), the position of which was indicated by the vertical dotted line in (d). The grey level was normalized by the signal amplitudes indicated by the two vertical dashed lines in (c). Each horizontal trace in the M-mode image indicated the transient displacement of the AC tissues at different depths, induced by the change in saline concentration. The solid and open triangle marks indicate the first positive half cycle of the reflection signal from the AC surface and that from the AC/bone interface, respectively.

Fig. 2. Diagram of the ultrasound swelling measurement system. The AC specimen was fixed on the bottom of the container, surrounded by rubber gel to prevent the solutions from penetrating from the sides of the specimen and submerged in saline solution. The ultrasound transducer could be moved in three dimensions to focus its beam at the center of the AC specimen.

Fig. 3. The M-mode representation of the ultrasound signals collected from a consistent site of AC specimen (#2) during the continuous monitoring for different processes. The

solid and open triangle marks indicate the first positive half cycle of the reflection signal from the AC surface and that from the AC/bone interface, respectively. The echoes from the AC surface were at the top. (a) 0.15 M saline replaced with 2 M saline; (b) 2 M saline replaced with 0.15 M saline; (c) 0.15 M saline replaced with 1 mg/ml trypsin; (d) trypsin replaced with 0.15 M saline; (e) 0.15 M saline replaced with 2 M saline after the trypsin digestion; (f) 2 M saline replaced with 0.15 M saline after trypsin digestion. The dashed line in (a) indicates that the penetration of 2 M saline could be observed from the disturbance of the ultrasound signals. Similar results could be observed in Figs. 2d and 4a-d for different specimens. The shifts in the echoes from the AC surface and those from the AC/bone interface were mainly due to a change in the thickness and ultrasound speed, respectively, of the AC. The inclined streaks in (c) and (d) indicate the progressive penetration of the trypsin digestion front. The radio-frequency signals at the positions indicated by the dashed lines A, B, and C in (b), (c) and (d) are drawn in Fig. 4.

Fig. 4. The radio-frequency ultrasound signals collected at the time points indicated by the dashed lines A, B, and C in Figs. 3(b), (c) and (d). The three time points represent the statuses of the specimen in 0.15 M saline before digestion, in the middle of the 6 h trypsin digestion and in the middle of the 3 h period after replacing the trypsin with 0.15 M saline. The dashed circles represent the additional echoes caused by the interface of the trypsin digested and undigested AC tissues. It appears that the progressive penetration of the trypsin digestion front did not affect the ultrasound scattering signals from the AC tissues.

Fig. 5. The M-mode representation of the ultrasound signals collected from different specimens with the solid and blank triangle marks indicate the first positive half cycle of the reflection signal from the AC surface and that from the AC/bone interface, respectively. (a) The results of normal specimen #3 after the solution was changed from 0.15 to 2 M of saline; and (b) The results of the same site of the same specimen with the same process as (a), but measured 10 h later. After the measurement of (a), the specimen went through the same experimental protocol as shown in Fig. 3, except that the trypsin solution was replaced with a 0.15 M saline solution. (c) The results of normal specimen #4 after the solution was changed from 0.15 to 2 M saline; and (d) the results of the same specimen with same process as (c) but digested by trypsin for 6 h. (c) and (d) were the results of two steps out of a whole procedure similar to that shown in Fig. 3, but the specimen was stored under -20ºC after the trypsin digestion. Thus, the specimen went through the same thawing process before the results were collected in (c) and (d). (e) The results of the dehydrated/hydrated specimen #5 after 0.15 M saline was replaced by 2 M; and (f) The results of the same specimen as (e) after 2 M saline was replaced by 0.15 M. This specimen was previously dehydrated and stored for eight months and was hydrated for 6 h before the test.

Fig. 6. The shifts of the ultrasound echoes reflected from the AC surface and the AC/bone interface extracted from the results of two consecutive tests for a specimen. In each test, the concentration of the saline solution was first changed from 0.15 M to 2 M and then back to 0.15 M. (a) and (b) Show the shifts of the echoes after the saline was changed

from 0.15 to 2 M, and from 2 to 0.15 M, respectively. Results showed that the obtained patterns of the time shifts of the echoes were repeatable for the two consecutive tests.

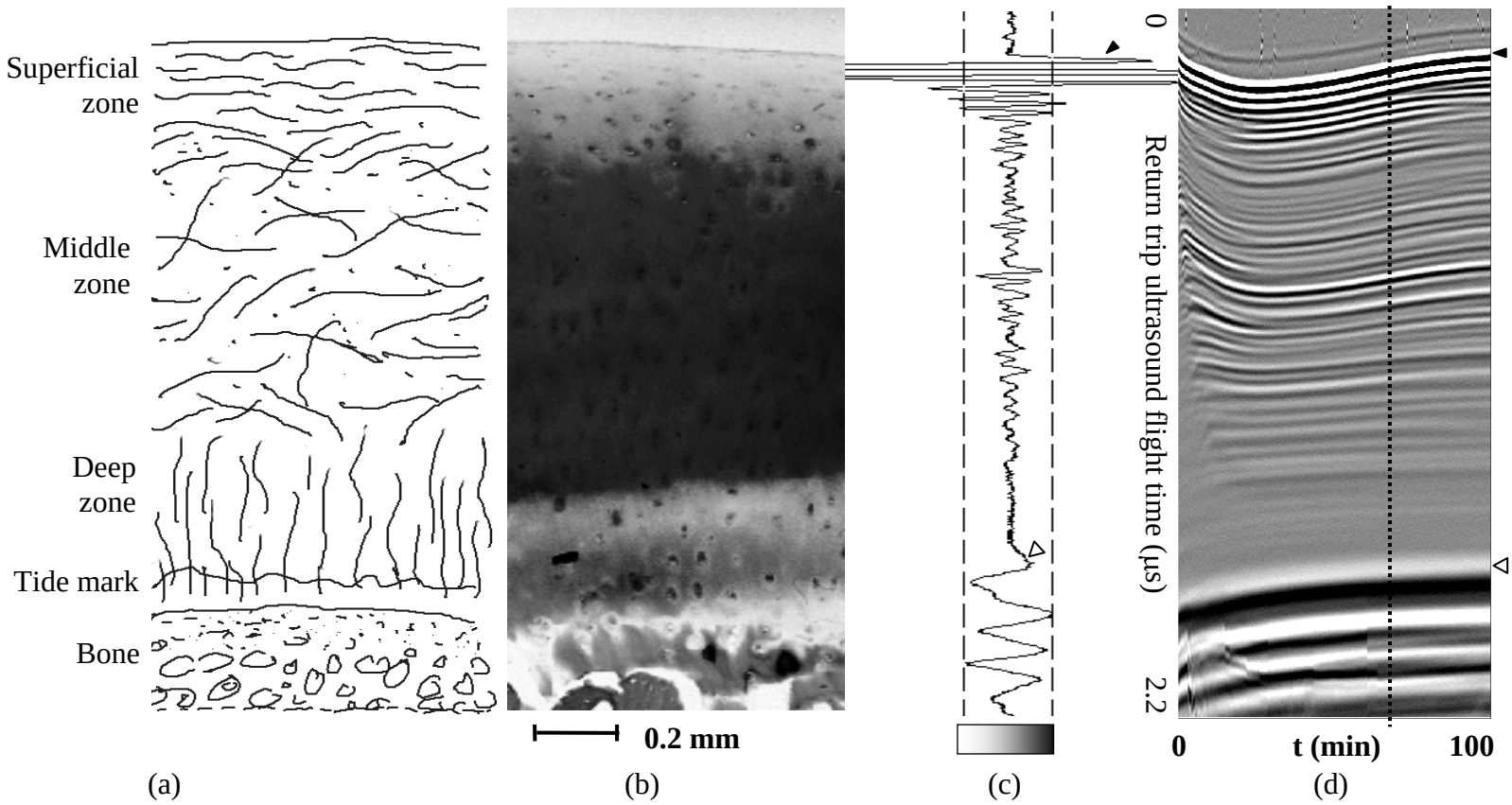

Superficial zone
Middle zone
Deep zone
Tide mark
Bone
0.2 mm
0
Return trip ultrasound flight time (μs)
2.2
0
t (min)
100
(a)
(b)
(c)
(d)

Figure 1.

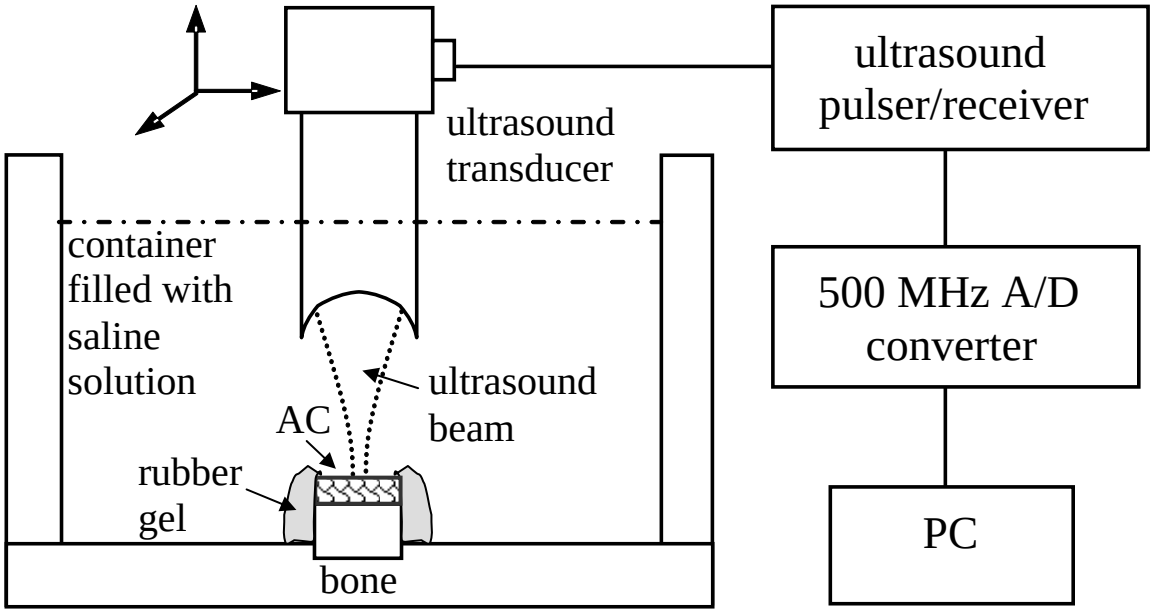

ultrasound
pulser/receiver
ultrasound
transducer
container
filled with
saline
solution
500 MHz A/D
converter
ultrasound
beam
AC
rubber
gel
PC
bone

Figure 2.

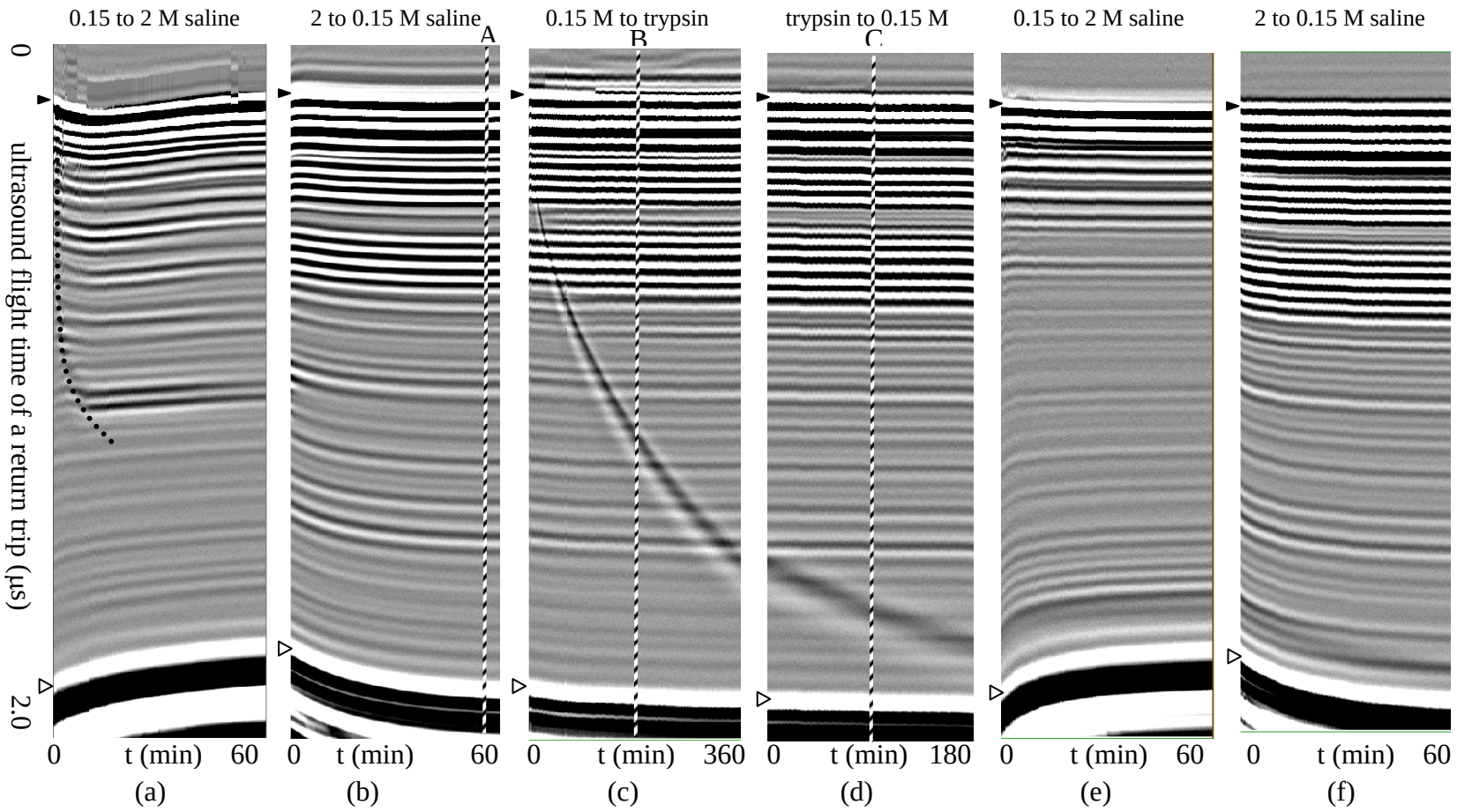

0.15 to 2 M saline
2 to 0.15 M saline
0.15 M to trypsin
trypsin to 0.15 M
0.15 to 2 M saline
2 to 0.15 M saline
A
B
C
0
2.0
ultrasound flight time of a return trip (μs)
0 t (min) 60
0 t (min) 60
0 t (min) 360
0 t (min) 180
0 t (min) 60
0 t (min) 60
(a)
(b)
(c)
(d)
(e)
(f)

Figure 3.

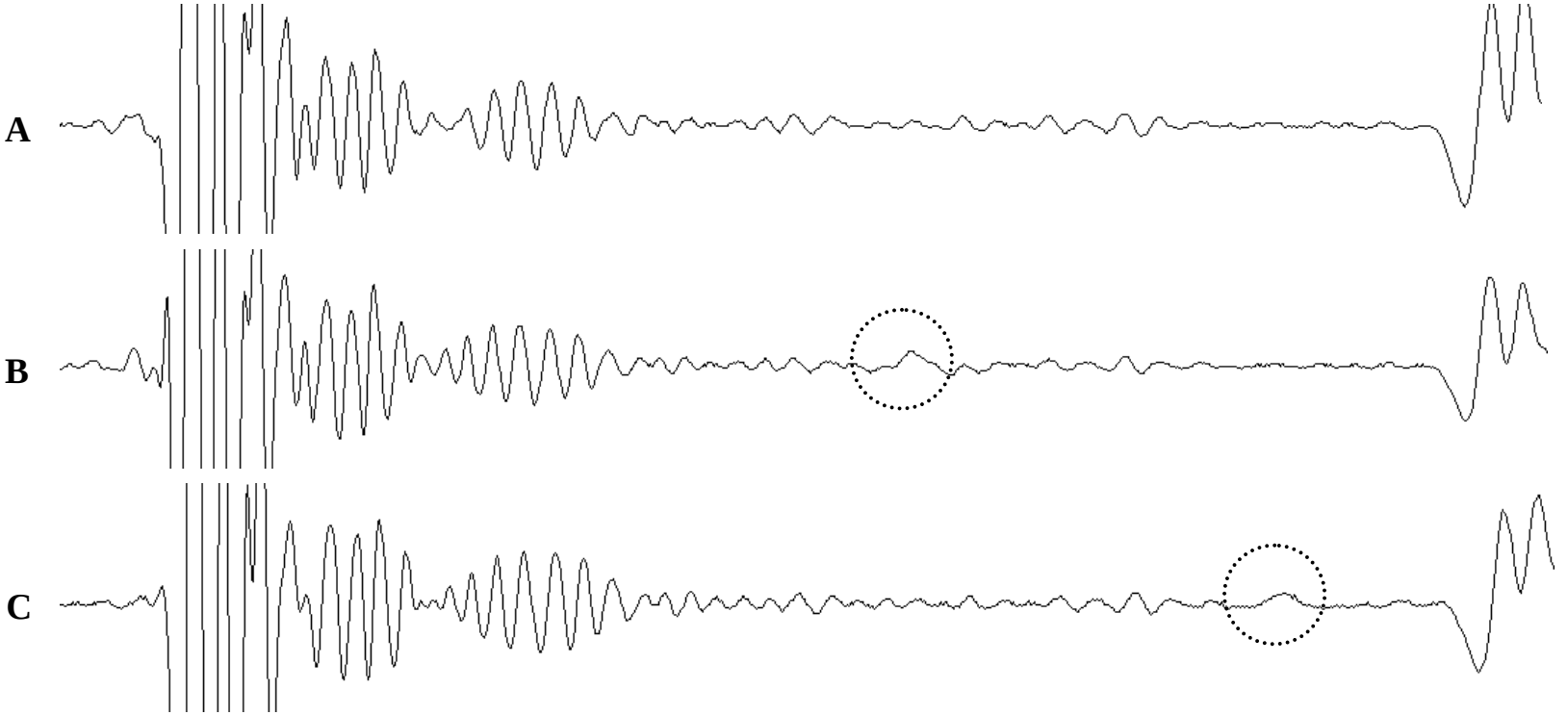


Figure 4.

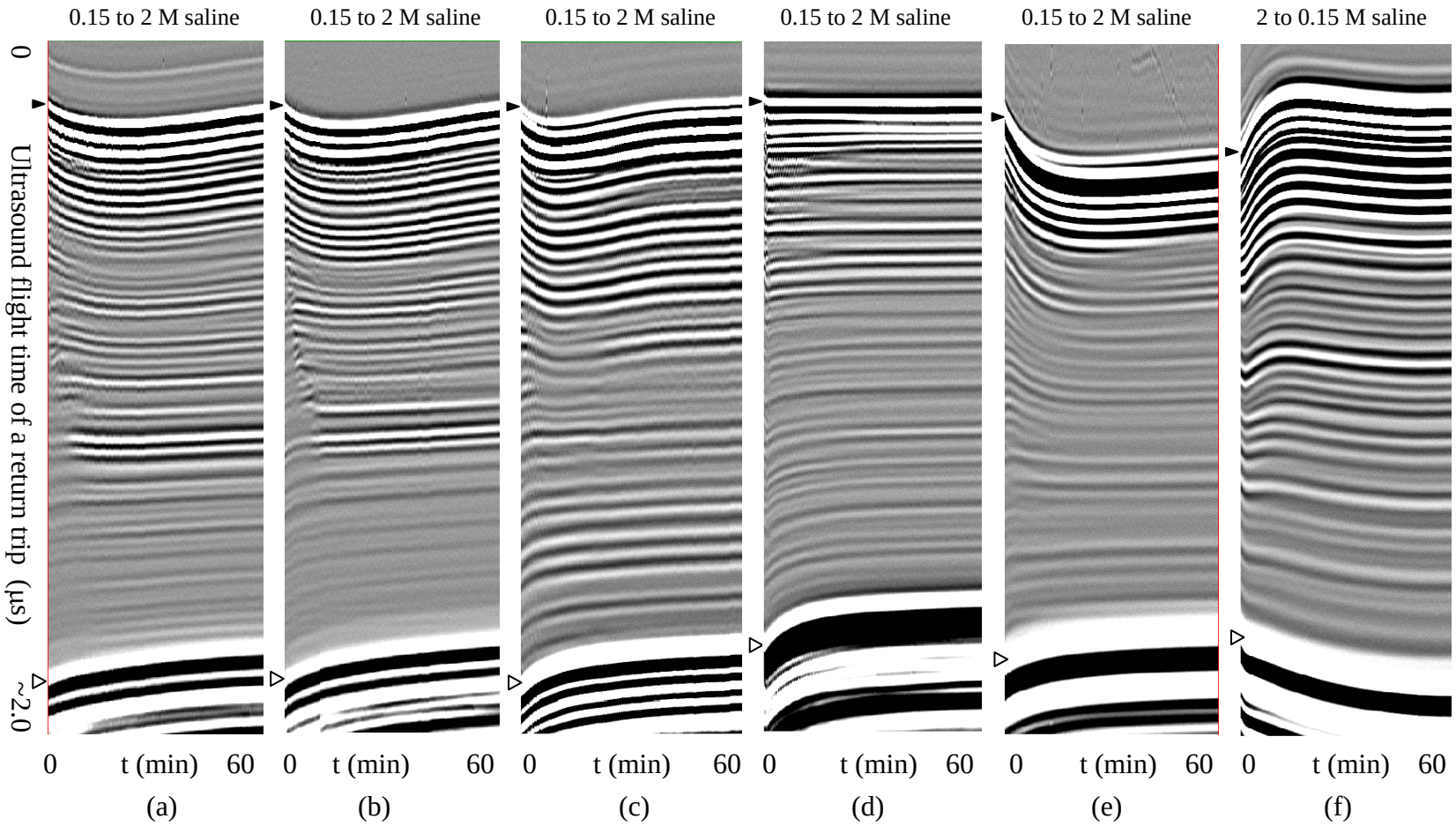
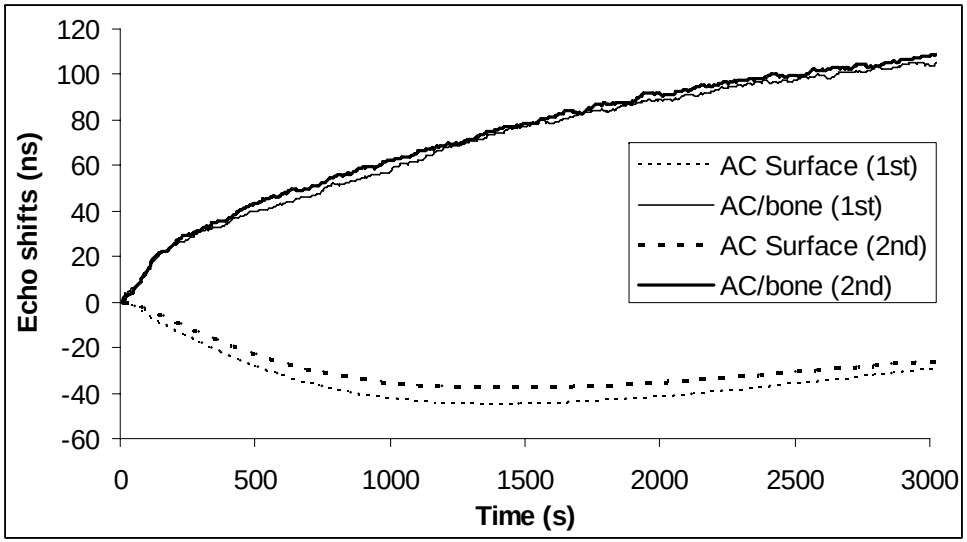
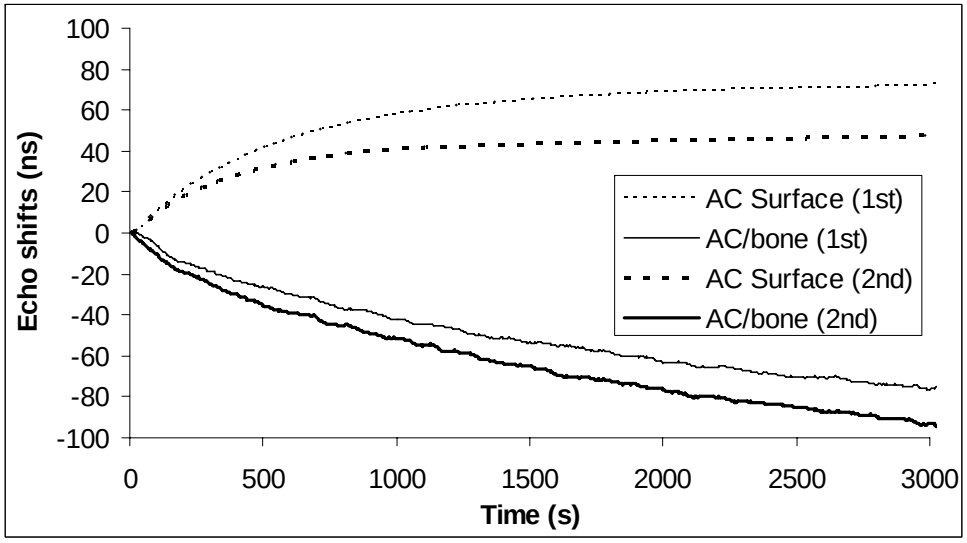

0.15 to 2 M saline
0.15 to 2 M saline
0.15 to 2 M saline
0.15 to 2 M saline
0.15 to 2 M saline
2 to 0.15 M saline
0
Ultrasound flight time of a return trip (μs)
~2.0
0
t (min)
60
(a)
(b)
(c)
(d)
(e)
(f)

Figure 5.


Echo shifts (ns)
120
100
80
60
40
20
0
-20
-40
-60
0
500
1000
1500
2000
2500
3000
Time (s)
AC Surface (1st)
AC/bone (1st)
AC Surface (2nd)
AC/bone (2nd)

(a)


Echo shifts (ns)
100
80
60
40
20
0
-20
-40
-60
-80
-100
0
500
1000
1500
2000
2500
3000
Time (s)
AC Surface (1st)
AC/bone (1st)
AC Surface (2nd)
AC/bone (2nd)

(b)

Figure 6.