

1 **Measurement of Depth-dependency and Anisotropy of Ultrasound**
2 **Speed of Bovine Articular Cartilage *In Vitro***

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4 Patil SG¹, Zheng YP¹, Wu JY², and Shi J¹
5

6 ¹ Rehabilitation Engineering Center, The Hong Kong Polytechnic University,
7 Kowloon, Hong Kong SAR, China
8

9 ² Department of Applied Biology and Chemical Technology, The Hong Kong
10 Polytechnic University, Kowloon, Hong Kong SAR, China
11

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13 **Short Running Title: Sound Speed of Articular Cartilage**
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17 Corresponding Author:

18 Dr. Yongping Zheng
19 Rehabilitation Engineering Center
20 The Hong Kong Polytechnic University
21 Kowloon, Hong Kong SAR
22 China
23

24 Tel: 852 276676674

25 Fax: 852 23624365

26 Email: htzheng@polyu.edu.hk
27
28
29

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1 Measurement of Depth-dependency and Anisotropy of Ultrasound 2 Speed of Bovine Articular Cartilage *In Vitro*

3 4 Abstract

5 The inhomogeneous and anisotropic mechanical properties and structural components
6 of articular cartilage (AC) may cause complex acoustic properties in this important
7 tissue. In this study, we used 50 MHz ultrasound to measure *in vitro* the depth-
8 dependence and anisotropy of the ultrasound speed of AC collected from the bovine
9 patellae. The ultrasound speeds of 18 disk AC specimens sampled from 18 different
10 patellae were measured in two orthogonal directions. One full-thickness layer (n = 18)
11 and three horizontal slices (n = 18*3) with approximately equal thickness were
12 prepared from each AC disk and measured with the ultrasound beam perpendicular to
13 the AC surface. One full thickness vertical slice (n = 10) was measured at different
14 depths with the ultrasound beam parallel to the AC surface. The measured ultrasound
15 speeds of AC in the two orthogonal directions were significantly different (p < 0.001).
16 The ultrasound speeds also significantly increased with the increase of tissue depth for
17 both measurement directions (p < 0.001). The ultrasound speeds of AC from the
18 superficial to deep regions were 1518±17 (Mean + SD), 1532±26 and 1554±42 m/s
19 for the ultrasound beam parallel to the AC surface, and 1574±29, 1621±34 and
20 1701±36 m/s for the beam perpendicular to the AC surface. The sound speed of the
21 full-thickness layer was 1636±25 m/s. The results suggested that the depth-
22 dependency and anisotropy of the ultrasound speed in AC should be taken into
23 account when ultrasound is used for the AC measurement. It was also demonstrated in
24 this study using additional specimens (5*2) that the swelling of AC after detaching
25 from its subchondral bone could cause the change of its sound speed.

26 **Key Words:** ultrasound speed, high-frequency ultrasound, articular cartilage, cartilage
27 biomechanics, osteoarthritis, cartilage degeneration

1 **Introduction**

2 Articular cartilage (AC) is a biphasic biological soft tissue that covers the end
3 of articulating bones within synovial joints. It mainly consists of an organic composite
4 matrix filled with liquids. Structurally, the organic composite matrix of AC can be
5 regarded as a proteoglycan gel reinforced by a network of fine collagen fibrils and
6 swollen with a multi-ionic electrolytic aqueous solution (Mankin et al. 1994; Mow et
7 al. 1991). The biomechanical properties of AC are mainly determined by the organic
8 composite matrix and its interactions with the liquid phase. Functionally, AC serves
9 mainly as load bearing tissue in joints to carry high stresses and plays essential roles
10 in joint lubrication (Kempson 1980; Mow et al. 1991). Fig. 1 illustrates the three
11 zones of AC and the dimensional distribution of collagen over its full thickness. The
12 volumetric concentration of collagen fibres increases from the superficial to deep
13 zone (Langsjo et al. 1999). It is well accepted that collagen and proteoglycans of AC
14 establish its tensile property and the compressive stiffness, respectively (Armstrong
15 and Mow 1982; Harkness 1968; Kempson et al. 1973; Laasanen et al. 2003a). Due to
16 the spatial variation of the water content, the proteoglycan concentration and the
17 orientation of the collagen fibrils, the mechanical properties of AC are different at
18 different depths. Therefore, AC is referred as structurally inhomogeneous and exhibits
19 anisotropic mechanical properties. Wu and Herzog (2001) reported that the
20 orientations of the collagen fibres and chondrocytes were responsible for the
21 anisotropic mechanical property AC. Some studies reported the anisotropy of AC in
22 compression (Jurvelin et al. 1996; Koehler et al. 2001; Wang et al. 2003). Measuring
23 the depth-dependent properties of AC is important not only for the investigation of
24 AC structure but also for finding the reason behind its degeneration as well as for the
25 tissue engineering of AC (Risbud and Sitterling 2002).

1 The change of AC thickness has been widely used as an indicator to its
2 degeneration status. The tissue thickness is also an important parameter for the
3 measurements of the modulus of AC using indentation (Hayes et al. 1972). The
4 thickness of AC was conventionally measured using calibrated microscopes (Jurvelin
5 et al. 1995; Myers et al. 1995), micrometer installed with microscopes (Modest et al.
6 1989), and needling techniques (Jurvelin et al. 1995; Swann and Seedhom 1989;
7 Toyras et al. 1999; Yao and Seedhom 1999). Most of the above techniques can only
8 be used *in-vitro*. The AC thickness can also be measured *in-vivo* using MRI
9 (McGibbon 2003) and X-ray (Buckland-Wright et al. 1995; Adam et al. 1998).

10 Recently, ultrasound techniques have been widely used for the measurement
11 of AC thickness with a high resolution. In addition, ultrasound has been used to
12 facilitate the measurement of biomechanical properties of AC using indentation or
13 compression tests (Chen and Sah 2000; Fortin et al. 2003; Laasanen et al. 2002;
14 2003b; Macirowski et al. 1994; Mann et al. 2001; Nieminen et al. 2002; Rushfeldt et
15 al. 1981; Toyras et al. 2001; Youn and Suh 2001; Zheng et al. 1998; 2001; 2002).
16 Despite of the wide use of ultrasound in the morphological and biomechanical
17 assessment of AC, ultrasound propagation in AC has not been well understood,
18 particularly for its depth-dependency and anisotropy.

19 The sound speed in AC is an important parameter for the measurement of AC
20 thickness using ultrasound (Adam et al. 1998; Lefebvre et al. 1998; Modest et al.
21 1989; Rushfeldt et al. 1981; Saied et al. 1997; Toyras et al. 2001). Constant
22 ultrasound speeds were frequently used in these studies based on the values reported
23 in the literature. Yao and Seedhom (1999) reported a very large variation of
24 ultrasound speed of human AC. In their study, the thickness was measured using the
25 needling technique. The accuracy of the sound speed measurement might be affected

1 by the mismatch of the exact measurement site or direction for the needle punching
2 and ultrasound reflection. Suh et al. (2001) measured the ultrasound speed and
3 thickness of AC tissues simultaneously *in situ* by applying an indentation on the AC
4 surface. Using this technique, the ultrasound speed of AC could be measured without
5 the knowledge of AC thickness. Since the stiffness of AC tissues significantly
6 depends on the depth (Guilak et al. 1995; Schinagl et al. 1996; Wang et al. 2002;
7 Zheng et al. 2001; 2002), AC is compressed non-uniformly during indentation. Thus
8 the knowledge about the depth-dependence of ultrasound speed of AC is critical to
9 this new approach.

10 The sound speed may vary in AC due its heterogeneous structure through its
11 depth. Agemura et al. (1990) measured sections of bovine AC prepared both parallel
12 and perpendicular to the AC surface using a scanning laser acoustic microscope with
13 an ultrasound frequency of 100 MHz. Their preliminary study with two specimens
14 demonstrated that the ultrasound speed of AC tissue measured with the ultrasound
15 beam parallel to the AC surface varied with its depth. In the present study, we
16 investigated the depth-dependent and anisotropic behaviors of the AC using a non-
17 contact ultrasound method, which measured the sound speed and thickness of the
18 specimen simultaneously. Similar methods have been used to obtain the sound speeds
19 of various engineering materials (Kuo et al. 1990; Hsu and Hughes 1992).

20

21 **Materials and Methods**

22 *Experimental Setup*

23 A schematic representation of the experimental setup is shown in Fig. 2. A
24 focused ultrasound transducer (Model PI50-2, Panametrics, Waltham, MA USA) with

1 a nominal frequency of 50 MHz, a focal zone diameter (-6 dB) of 0.1 mm, and a focal
2 length of 12.8 mm was fixed to a positioning stage (Model R301MMX, Deltron
3 Precision Inc., Bethel, CT). The ultrasound transducer could be translated vertically in
4 one dimension with a precision of 0.01 mm. The specimen was installed on a pair of
5 plates and secured by two elastic threads to prevent potential movements during the
6 test. Three pairs of this arrangement were contained in a saline bath which could be
7 translated in two horizontal directions with a precision of 0.01 mm via a positioning
8 stage (Model 2201MMXY, Deltron Precision Inc., Bethel, CT). The focus of the
9 ultrasound transducer was located approximately at the middle of the specimen.

10 The ultrasound transducer was driven by an ultrasound pulser/receiver (Model
11 5601A, Panametrics, Waltham, MA USA). The received ultrasound signals were
12 digitized by an A/D converter (CompuScope 8500PCI, Gage, Canada) at a sampling
13 rate of 500 MHz, and stored in the PC for offline signal analysis (Zheng and Mak
14 1996; Zheng et al. 2002). The pulser/receiver settings, including the transmitting
15 energy, receiving gain, attenuation and damping were selected according to pilot
16 experiments and were maintained during the course of the experiments.

17 *Specimen Preparation*

18 Twenty-three fresh mature bovine patellae without any surface damage were
19 harvested within 6 h of death and stored in a refrigerator at -20°C prior to sectioning.
20 It has been reported that cryopreservation (Kiefer et al 1989), freezing and thawing
21 (Agemura et al 1990; D'Astous and Foster 1986; Kim et al 1995) of specimens would
22 not affect the mechanical and acoustic properties such as ultrasound speed,
23 attenuation and backscatter of AC. Specimen preparation was carried out by excising
24 the lateral part of the patella, and then separating it into two pieces by cutting
25 perpendicular to the articular surface. An AC slab with a bone layer approximately 5

1 mm thick was prepared from each piece, and 6.35 mm plugs were cored out from the
2 flat area of slab using a metal punch as shown in Fig. 3. The samples were kept moist
3 with normal saline solution during preparation. One specimen from the upper right
4 quadrant of each patella was tested in this study after further preparations described as
5 follow.

6 Three different categories of AC slices were prepared from 18 disks in the second
7 phase of specimen preparation (Fig. 4). A thin surgical blade was used manually to
8 excise a small portion of AC with a width of approximately 2 mm for full-thickness
9 measurement (n = 18). A lateral vertical slice with a width of approximately 1 mm
10 was then prepared (n = 10), and the AC surface was marked as an indicator for this
11 block. The remaining portion of the AC was cut into three horizontal slices (n = 18*3)
12 parallel to the AC surface, each of approximately equal thickness. These three groups
13 of specimens were then used for the ultrasound measurement within 15 minutes after
14 the preparation.

15 The remaining 5 disks were prepared to investigate the swelling effects of AC
16 after the specimens were detached from the bone. The AC disks were first thawed in
17 saline for at least 2 h before further preparation. A full-thickness slice was first
18 excised from each disk and installed onto the specimen platform for measurement
19 with the ultrasound beam perpendicular to the AC surface. The procedure for the
20 specimen preparation and installation was completed within 1 min. After the test of
21 this specimen, another full-thickness AC specimen was prepared from the same AC
22 disk. The procedure was similar as that of the first full-thickness slice, however the
23 AC slice was obtained from the diametrically opposite direction of the disk to avoid
24 the potential effects caused by the previous cut. This specimen was installed with the
25 ultrasound beam parallel to the AC surface.

1

2 *Measurement Procedure*

3 *Slice Measurement for Sound Speed.* The vertical slice and full-thickness
4 slices were first placed on the plates in the container. The superficial layer of the full-
5 thickness slice was arranged to face the ultrasound beam. In the case of vertical slice
6 it was made sure that the superficial layer was scanned first followed by the middle
7 and deep layers with the ultrasound beam parallel to the AC layers. The ultrasound
8 beam was first located approximately 2 mm away from the edge of the first AC
9 specimen and ultrasound signal reflected from the bottom of the container was
10 collected. Then the ultrasound transducer was moved horizontally towards the
11 specimen in 0.25 mm intervals and a total of eight sites on the container bottom were
12 measured. The ultrasound beam was then moved to pass through the vertical slice
13 specimen and ultrasound signals were recorded at intervals of 0.05 mm. The collected
14 ultrasound signals included echoes from the upper and lower surfaces of the specimen,
15 and the bottom of the container. As shown in Fig. 4, the ultrasound beam was scanned
16 along the vertical cutting interface of the AC specimen and measurements were made
17 on a number of sites located from the superficial layer to the deep layer. The position
18 of each measurement site was recorded from the micrometer screw gauge. After the
19 ultrasound beam passed the specimen region, ultrasound signals were collected from
20 the gap between the vertical slice and the full-thickness slice at intervals of 0.25 mm.
21 Similarly, ultrasound signals from the full-thickness slice were collected at a distance
22 of 0.05 mm. Beyond the full-thickness specimen, ultrasound signals were recorded
23 again at eight sites on the bottom of the container with 0.25 mm intervals. Three scans
24 were performed across each specimen at a distance of 0.5 mm (Fig 4).

1 The horizontal AC slices with the cut parallel to the AC surface were
2 measured in a similar way. Three slices from a single specimen were placed on the
3 three pairs of plates in the container. The ultrasound beam was scanned along the
4 container bottom at equal intervals of 0.25 mm and along the specimens at equal
5 intervals of 0.05 mm. An example of the ultrasound reflections from the upper and
6 lower surfaces of the specimen and the surface of the container base is shown in Fig.
7 5.

8 ***Baseline Drift Amendment.*** The perpendicular orientation of the ultrasound
9 beam to the container bottom is important for accurate measurements. To ensure the
10 uniformity of the distance from the transducer to the container bottom at different
11 scanning sites, the baseline of the container bottom was first carefully adjusted before
12 the measurement. In addition, further correction was made by using the ultrasound
13 echoes reflected directly from the bottom of the container on the measurement sites
14 and beyond the AC specimens. A pair of tracking cursors was used to track the shift
15 of the echoes reflected from the bottom of the container directly or passing through
16 the AC specimen. Since the horizontal positions of the measurement sites were
17 recorded with the micrometer screw gauge, a linear regression between the position
18 and the flight time shift was made for the measurement sites without the presence of
19 AC specimen. The regression line represented the slope of the container bottom and
20 was used to correct the flight time of the echoes reflected from the bottom of the
21 container for the measurement sites on the AC specimen.

22 ***Calculation of Ultrasound Speed in Saline and AC.*** Ultrasound reflections
23 were collected by a program in real time and were analyzed offline. As shown in Fig.
24 5, three pairs of tracking windows were used to track the signals reflected from the
25 interfaces of the specimen and the container. Each tracking cursor could be moved

1 individually with a time resolution of 2 ns as defined by the sampling rate (500 MHz)
2 of the A/D converter. In this study, cross-correlation of the ultrasound echoes was
3 used as references for the measurement of flight time T . Two corresponding full
4 cycles of RF signal were marked by the tracking windows on the echoes reflected
5 from two interfaces of AC slices, respectively. Fig. 6 shows a schematic
6 representation for the ultrasound paths and flight times involved in the calculation of
7 the ultrasound speeds. T_1 , T_2 , and T_3 represent the flight times of the round trips of
8 ultrasound from the transducer to the interfaces of the upper surface of the AC slice,
9 the lower surface of AC slice, and the bottom of the container through the specimen.
10 T_4 represents the round trip from the bottom of the container without the presence of
11 the specimen. The ultrasound speed in the saline solution was measured by moving
12 the transducer vertically down by two steps of 0.5 mm and then back to the original
13 position in two steps. T_5 represents the position of the transducer after it is moved
14 from its original position. The difference between the flight times obtained at the four
15 positions was used to calculate the ultrasound speed in the saline solution as follow:

$$16 \quad c_w = \frac{2d_T}{(T_4 - T_5)} \quad (1)$$

17 where c_w is the ultrasound speed in saline, d_T was the vertical distance traveled by
18 the transducer. An average value of the speed in the saline solution calculated for all
19 the steps was used for the further calculation of the ultrasound speed in AC.

20 The ultrasound speed in AC was calculated as follows (Refer to Fig. 6):

$$21 \quad \frac{T_3}{2} = \frac{d_{ab}}{c_w} + \frac{d_{bc}}{c_{AC}} + \frac{d_{cd}}{c_w} \quad (2)$$

$$22 \quad \frac{T_4}{2} = \frac{d_{ab}}{c_w} + \frac{d_{bc}}{c_w} + \frac{d_{cd}}{c_w} \quad (3)$$

1 Also

$$2 \quad \frac{T_1}{2} = \frac{d_{ab}}{c_w} \quad (4)$$

$$3 \quad \frac{T_2}{2} = \frac{d_{ab}}{c_w} + \frac{d_{bc}}{c_{AC}} \quad (5)$$

$$4 \quad \frac{d_{cd}}{c_w} = \frac{T_3}{2} - \frac{T_2}{2} \quad (6)$$

5 d_{ab} , d_{bc} , and d_{cd} represent the distance between the transducer and the specimen
6 upper surface, the specimen thickness, and the distance between the specimen lower
7 surface and the surface of the container base, respectively. By substituting eqns (4)
8 and (6) into eqns (2) and (3), we get:

$$9 \quad d_{bc} = \left(\frac{T_2}{2} - \frac{T_1}{2} \right) * c_{AC} \quad (7)$$

$$10 \quad \frac{T_4}{2} = \frac{T_1}{2} + \frac{d_{bc}}{c_w} + \frac{T_3}{2} - \frac{T_2}{2} \quad (8)$$

11 By further substituting eqn (7) into eqn (8), we get:

$$12 \quad c_{AC} = \frac{T_4 - T_3 + T_2 - T_1}{T_2 - T_1} * c_w \quad (9)$$

$$13 \quad d_{AC} = c_{AC} * \frac{T_2 - T_1}{2} \quad (10)$$

14 where c_{AC} is the ultrasound speed in the AC tissue and d_{AC} is the thickness of AC
15 slice.

16 **Measurement for Swelling Effects.** It was a concern whether the sound speed of
17 AC would change as a function of time due to swelling effects after it was detached
18 from its subchondral bone. The two full-thickness slices prepared from each of the 5

1 patellae were immediately installed onto the specimen platform and monitored for 1 h
2 with the ultrasound beam parallel and perpendicular to the AC surface, respectively.
3 The monitoring site was located at approximately the middle of the surface facing the
4 ultrasound beam. The transient thickness and sound speed of the AC specimen were
5 calculated using the method introduced in the last section, where T_4 in eqn (9) was
6 measured after the specimen was removed from the specimen platform.

7 **Data Analysis.** The ultrasound speeds measured at the various sites, along the
8 three scanning lines were averaged to obtain a mean value for the full-thickness and
9 horizontal slices. This averaged value was used for the further data analysis of the
10 superficial, middle and deep slices. For the vertical slice, the ultrasound speeds
11 measured at different sites along a scanning line was equally separated into three
12 groups representing superficial, middle and deep regions. The ultrasound speeds of
13 the measurement sites of each region were averaged, and the result was further
14 averaged with those obtained along the other two scanning lines.

15 Two-factor ANOVA (SPSS v11.0.0, SPSS Inc., Chicago, US) was used to test
16 the significance of the differences in ultrasound speeds measured for the three
17 horizontal slices and the three regions of the vertical slices. Both the region
18 dependence and the orientation dependence of the ultrasound speed in AC tissues
19 were tested. In order to test the reproducibility of the technique, the sound speeds
20 measured along the three scan lines (0.5 mm apart) for each slice were compared
21 using intra-class correlation (SPSS v11.0.0, SPSS Inc., Chicago, US). The mean of
22 the percentage standard deviation for the three measurements of each slice was also
23 calculated as an indicator for reproducibility.

24
25

1 **Results**

2 The reproducibility test showed the sound speed measured along the three
3 different scanning lines for each slice agreed well with a mean percentage standard
4 deviation of 2.2%. The intra-class correlation coefficient for 95% confidence level
5 was $r = 0.9721$ ($p < 0.001$), which showed a very high reproducibility.

6 The mean thickness (+SD) of the superficial, middle and deep horizontal slices
7 ($n = 18 \times 3$) were 0.64 ± 0.18 mm, 0.50 ± 0.18 mm, and 0.59 ± 0.18 mm, respectively.
8 The mean thickness for the full-thickness layers ($n = 18$) was 1.64 ± 0.30 mm, which
9 was slightly smaller than the sum of the three slices (1.73 ± 0.31 mm). The increase
10 (5.5%) of the summed thickness of the slices was possibly due to the AC swelling
11 after slicing. This swelling issue would be further discussed in the following sections.

12 The ultrasound speeds of AC tissues at the superficial, middle and deep
13 regions were 1518 ± 17 m/s (Mean \pm SD), 1532 ± 26 m/s and 1554 ± 42 m/s with the
14 ultrasound beam parallel to the AC surface ($n = 18$), and 1574 ± 29 m/s, 1621 ± 34 m/s
15 and 1701 ± 36 m/s with the beam perpendicular to the AC surface ($n = 10$) (Fig. 7).
16 The ultrasound speeds of AC tissue significantly increased from the superficial to
17 middle and deep regions for both measurement directions (ANOVA, $p < 0.001$). The
18 ultrasound speeds of AC measured in the two orthogonal directions were significantly
19 different (ANOVA, $p < 0.001$). There was a quadratic relationship ($R^2 = 0.9982$)
20 between the ultrasound speed and the tissue depth for the measurements of the
21 vertical slices (Fig. 8). The error bars in Fig. 8 represents the standard deviation
22 among the results of the 10 specimens. The variation among the specimens as shown
23 in Fig. 8 was similar to those in Fig. 7, though the scale was changed in Fig. 8 better
24 to represent the quadratic relationship. The sound speed increased from 1518 m/s to
25 1559 m/s (2.7%) when the measurement region moved from the most superficial to

1 the deepest zone of the AC layer. The sound speed of the full-thickness AC layer (n =
2 18) was 1636 ± 25 m/s (ranged from 1598 m/s to 1721 m/s).

3 It was demonstrated that there was an increasing trend for the AC thickness
4 for both measurement directions for the 5 specimens tested (Fig. 9a). No distinguished
5 difference was noted between the results measured in the two directions. Hence the
6 results shown in Fig. 9a were the combined data of all the 10 slices from the 5
7 specimens. This change of the AC thickness was believed to be caused by the
8 swelling effect of AC due to its excision from the subchondral bone. Most of the
9 increase in thickness was observed within the first 5 min. At time points 1, 15, 30 and
10 60 min, the mean increases of the thickness were summarized in Table 1. The AC
11 thickness changed by 3.6% after 15 min. It appeared that the measurement performed
12 at 15 min was just slightly different in comparison with that performed at 60 min
13 (changed by 0.2%). Large variations among specimens were observed. Considering
14 the results of individual specimens, most of them showed different degrees of increase
15 but few of them showed slight decrease as time going.

16 The sound speed results showed similar but smaller increasing trend in
17 comparison with that of AC thickness (Fig. 9b). No distinguished difference was
18 observed for the changes of the sound speed between the results obtained in two
19 different directions. Most of the changes also happened within 5 min. At time points 1,
20 15, 30 and 60 min, the mean increases of the sound speed were also summarized in
21 Table 1. The sound speed changed by 1.2% after 15 min. It appeared that the
22 measurement made at 15 min were just slightly different in comparison with that
23 made at 60 min (changed by 0.2%). Considering the results of individual specimens,
24 majority of them showed different degrees of increase but some of them showed
25 decrease as time going.
26

1

2 **Discussion**

3 This paper reported a study of the depth-dependent and anisotropic behavior
4 of ultrasound speed in bovine patellar AC. It was demonstrated that the ultrasound
5 speed of AC significantly depended on the tissue depth. The ultrasound speed
6 measured with the ultrasound beam parallel and perpendicular to the AC surface both
7 increased from the superficial to the deep layer. The ultrasound speed measured with
8 the beam perpendicular to the AC surface (overall mean for different depths 1629 ± 71
9 m/s) (Mean + SD) was significantly larger than that measured with the beam parallel
10 to the AC surface (1535 ± 18 m/s). Similar results have been reported previously with
11 fewer specimens (Agemura et al 1990). The increasing trends of ultrasound speed in
12 both directions might be related to depth dependent mechanical properties and
13 structural components of AC. Similarly, the anisotropic behavior of the sound speed
14 in AC might be due to the anisotropic mechanical properties and micro-structures of
15 AC (Kempson et al. 1980, Mow et al. 1991, Mankin et al. 1994). The ultrasound
16 speed of the full-thickness AC was 1636 ± 25 m/s, which was within the similar range
17 of the averaged ultrasound speed of the three horizontal slices (1629 ± 71 m/s). The
18 sound speed measured in the present study with the 50 MHz ultrasound beam
19 perpendicular to the AC surface and under room temperature was within the similar
20 range as reported in most of the previous studies for bovine and human cartilages. The
21 sound speeds of full-thickness AC reported in the previous studies have been
22 summarized in Table 2. It was noted that most of the ultrasound speed of AC reported
23 in the literature was measured under either room temperature or 37°C . The effects of
24 temperature on the ultrasound speed measurement of AC deserve to be further studied.

1 It was noted that the waveforms of the ultrasound echo would distort for
2 different degrees as it propagates through the AC specimen. This distortion was
3 caused by the AC's frequency-dependent attenuation (Joiner et al. 2001) and possibly
4 nonlinear acoustic properties (Zheng et al. 1999). In this study, a cross-correlation
5 algorithm was used to measure the time-of-flight of the ultrasound signal in AC by
6 matching the RF wave of the echoes reflected from different interfaces. The distortion
7 of the echoes could potentially affect the cross-correlation matching (Ragozzino
8 1981). High correlations ($R^2 > 0.9$) between the echoes were achieved for most of the
9 specimens in this study. When the waveforms were significantly distorted, the echoes
10 to be matched were first manually located and the cross-correlation approach was then
11 used to optimize the matching.

12 The theoretical relationship between the longitudinal ultrasound speed and
13 mechanical parameters is shown in eqn (11).

$$14 \quad c = \sqrt{(K + 4/3G)/\rho} \quad (11)$$

15 where c is the longitudinal ultrasound speed, K is the bulk modulus, G is the
16 shear modulus, and ρ is the density of the material (Stanley 1968). Eqns (12) and (13)
17 provide the relationship between the bulk modulus K , shear modulus G , Young's
18 modulus E , and Poisson's ratio ν of an isotropic material (Mow et al 1991).

$$19 \quad K = \frac{E}{3(1 - 2\nu)} \quad (12)$$

$$20 \quad G = \frac{E}{2(1 + \nu)} \quad (13)$$

21 It has been reported that the Young's modulus of AC tissues increased from
22 superficial layer to the deep layer (Guilak et al. 1995; Laasanen et al. 2003a; Schinagl
23 et al. 1996; Wang et al. 2002; Zheng et al. 2002). The increasing trend of the
24 ultrasound speed of AC at depths from the AC surface to the bone appeared to agree

1 with increasing trend of equilibrium Young's modulus. Recently, Laasanen et al.
2 (2003a) reported that the middle region of the bovine knee AC had much larger
3 Poisson's ratio (up to 0.4) in compression as compared to the superficial and deep
4 regions (low to 0.1). Wang et al. (2003) demonstrated that the equilibrium
5 compressive moduli of AC were significantly different when measured in different
6 directions. Their results showed that modulus of the superficial region measured with
7 the compressing direction perpendicular to the AC surface was significantly smaller
8 than that measured with the compression in other two orthogonal directions. However,
9 the deep region of AC showed a reversed feature. Due to the complexity of
10 mechanical properties of AC, it appears that the correlation between the anisotropy
11 and depth-dependence of the equilibrium compressive modulus of AC and its
12 longitudinal ultrasound speed was not so straightforward. In addition to Young's
13 modulus and Poisson's ratio the contents of the fluid phase and the shear modulus of
14 AC are not constant from the superficial region to the deep region (Athanasίου et al.
15 1991). Even though there are few studies reported on the density of AC (Joseph et al.
16 1999), it is reasonable to predict that AC density is also depth-dependent considering
17 the depth-dependent water contents and other components. Considering the variations
18 of these parameters, it appears difficult to have a simple relationship between the
19 depth-dependent Young's modulus and the ultrasound speed.

20 Another difficulty is that material properties obtained in conventional
21 mechanical tests could not be directly used for eqn (11). According to the AC density
22 (1050 kg/m³) reported by Joseph et al. (1999), the instantaneous Young's modulus
23 (8.5 MPa, Laasanen et al. 2003, measured with 2 mm/s compression rate for 10%
24 deformation), and the instantaneous Poisson's ratio of 0.4 for bovine patellar AC
25 (Fortin et al. 2003, measured with a 2 s ramp compression); the sound speed

1 calculated using eqn (11) is only 132 m/s. This value was much smaller than that
2 measured in the present and all the previous studies. The main reason might be that
3 AC is a viscoelastic (biphasic poroelastic) material so that it is difficult to measure its
4 real instantaneous modulus and Poisson's ratio, which is involved in the acoustic
5 wave propagation as well as in eqn (11). The compression rate for measuring the
6 instantaneous modulus and Poisson's ratio as reported is much smaller as compared
7 with that happens in the ultrasonic wave. In other words, the real instantaneous
8 modulus of AC should be much larger than those reported in the literature. Other
9 factors like anisotropy and inhomogeneity of AC might also affect the result in using
10 eqn (11).

11 Toyras et al. (2003) reported that the ultrasound speed of full-thickness AC
12 significantly depends on the equilibrium Young's modulus, water content and other
13 AC composition. Agemura et al. (1990) reported that the differences in ultrasound
14 speed among different regions of AC to its dissimilar fibril organization. In the
15 superficial, middle and deep region, the collagen fibers are parallel, random and
16 perpendicularly oriented to the surface of the AC, respectively (Mankin et al. 1994;
17 Mow et al. 1991). Agemura et al. (1990) concluded with a limited number of
18 specimens that the ultrasound appeared to propagate faster across the long axis of
19 collagen fibrils than along them. In the present study the measured ultrasound speed
20 significantly increased from the superficial region to the deep region. A study with
21 fine slice preparation is necessary to further clarify the correlation between the
22 collagen fibril orientation and the ultrasound speed in AC.

23 Previous studies have suggested that detaching the AC from its subchondral
24 bone could cause swelling and curling of the specimen (Myers et al. 1984; Setton et al.
25 1998). We concluded from the results of the swelling tests that the swelling effects

1 after detaching should be taken into account during the measurement of the AC sound
2 speed and thickness. It was noted that the change of the speed of sound was minor
3 (1.2% at 15 min after detaching from the bone) but not negligible considering the
4 small changes caused by the variations of tissue depth and measurement directions.
5 The sound speed increased by 3.0% and 5.0% from the superficial to deep layer with
6 the ultrasound beam perpendicular to the AC surface; increased by 1.0% and 1.4%
7 with the beam parallel to the AC surface; and decreased by 6.2% when the
8 measurement direction was changed from perpendicular to parallel orientation to the
9 AC surface. In comparison with the variation of the sound speed among specimens,
10 the variation of sound speed caused by the detaching was found to be much larger.
11 The reasons for this large variation should be further studied together with the other
12 issues such as the variation during the period of 1 min while the specimen was
13 detached from bone and installed on the specimen holder.

14 It is obvious that the swelling effects to the sound speed should be taken into
15 account when AC is detached from the bone, particularly for the measurement with
16 the ultrasound beam parallel to the AC surface, where the change of the sound speed
17 as a function of tissue depth was relatively small. In spite of the fact that our results of
18 the depth- and orientation-dependent ultrasound speed might be affected by the
19 swelling effects explained above, they should still have reference values considering
20 the fact that majority of the sound speed measurement previously reported were based
21 on specimens detached from the subchondral bone. In addition, it appears that
22 measuring in-situ depth- or orientation-dependent ultrasound speed is still difficult
23 using available technology. More systematic studies on swelling effects are suggested
24 so that the outcome may benefit the field of ultrasonic and biomechanical
25 measurements of AC. Our preliminary results using 5 pairs of specimens

1 demonstrated that there was no obvious difference between the swelling effects
2 measured in two orthogonal directions. This finding should be supported by tests on a
3 larger number of specimens. The tissue depth may play an important role for the
4 swelling, as AC has depth-dependent composition, structure and mechanical property.
5 This issue should be addressed in the future studies. Without this information, the
6 conclusion of the depth-dependency of the sound speed of AC obtained in the present
7 study can be applicable to the AC specimens detached from the subchondral bone.
8 The depth-dependency of the swelling-induced change of the sound speed in AC is
9 not yet evident and requires further investigations.

10 In summary, the anisotropy and depth-dependency of the sound speed of AC
11 was investigated in this study for bovine patella specimens detached from the
12 subchondral bone using a noncontact ultrasound method that could obtain the speed of
13 sound and tissue thickness simultaneously. This noncontact measurement could be
14 very useful for accurate measurement of AC thickness and sound speed in future
15 ultrasonic studies for AC. The anisotropic nature of AC tissue was demonstrated as
16 the ultrasound speeds of AC measured in the two orthogonal directions were
17 significantly different. The ultrasound speeds of AC significantly increased from the
18 superficial to deep layers for both measurement directions. The influences of the
19 depth-dependent swelling effects to the depth-dependency of the sound speed in AC
20 need to be further studied. This study suggests that the depth-dependence and
21 anisotropy of ultrasound speed in AC tissues should be taken into account in the
22 studies for ultrasound characterization of AC. Although the measurements in the
23 present study were made on the normal bovine patellar AC, the results should be also
24 applicable for human AC because of their similar structure and biomechanical
25 properties. In the ultrasonic measurement of sound speed and thickness of AC

1 specimens, their orientations relative to the ultrasound beam should be carefully
2 marked and the measured AC zones should be documented. In cases of in-situ or in-
3 vivo measurement or imaging of the non-uniform mechanical or acoustic properties of
4 AC using ultrasound, the effect of depth-dependent sound speed should be
5 compensated or discussed.

6 **Acknowledgements**

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8 (PolyU5199/02E) and The Hong Kong Polytechnic University (G-T468).

9

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1 **Figure Captions**

2 **Fig. 1.** Schematic representation of the layered structure of AC (Mow et al. 1991).

3 **Fig. 2.** Experimental setup for the measurement of the ultrasound speed of AC. The
4 ultrasound transducer could be moved vertically to focus the ultrasound beam in the
5 specimen. The specimen base could also be moved horizontally in two dimensions to
6 locate different measurement points. The ultrasound signals were digitized, displayed
7 in real-time, and stored in PC for further off-line analysis.

8 **Fig. 3.** Schematic representation of the procedure for the preparation of AC plugs of
9 6.35 mm diameter. The specimens would be further prepared for the measurement of
10 the ultrasound speed of AC (Fig. 4).

11 **Fig. 4.** Schematic representation of the preparation of the AC cylinder into slice
12 specimens for ultrasound measurements. The full-thickness slice was removed first.
13 The vertical cutting separated the middle piece (vertical slice) used for the
14 measurement with the ultrasound beam parallel to the AC surface. The remained
15 larger piece was further cut into three pieces (horizontal slices) with approximately
16 equal thickness and used for the measurement with the ultrasound beam perpendicular
17 to the AC surface. The dots on each slice indicate the sites for the ultrasound
18 measurement.

19 **Fig. 5.** Typical ultrasound echoes from interfaces. A cross-correlation technique was
20 used to track the echoes. The echoes indicated by 'c' represents the reference signal
21 and echoes indicated by 'a' and 'b' were located by the cross-correlation tracking.
22 The echoes to be matched were overlapped with the reference echo in 'a' and 'b'.

23 **Fig. 6.** Schematic representation for the elements involved in the calculation of the
24 ultrasound speed in AC and saline. d_{AC} is the thickness of AC slice; T_1 to T_5 are the

1 flight times of the round trips of ultrasound from the transducer to different interfaces.
2 T_4 is measured after a vertical movement of the transducer and used to calculate the
3 ultrasound speed in the saline solution.

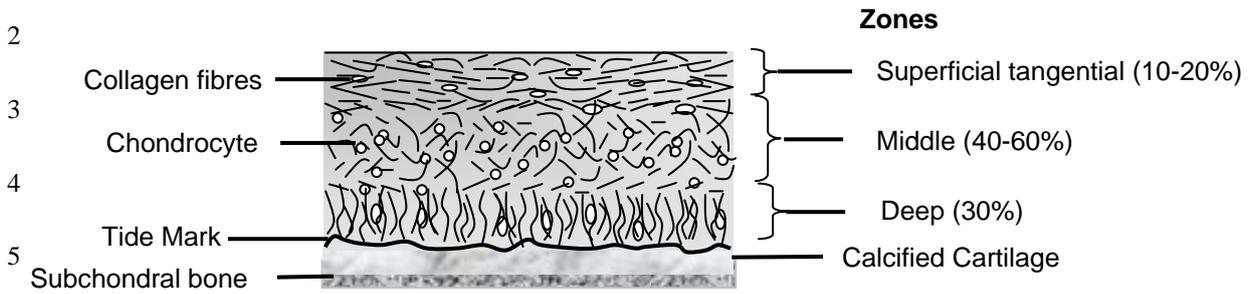
4 **Fig. 7.** Depth-dependent ultrasound speeds of AC tissues. The error bar represents the
5 standard deviation. The vertical slice was measured with the ultrasound beam parallel
6 to the AC surface, while the horizontal and full-thickness slices was measured with
7 the beam perpendicular to the AC surface. Two-factor ANOVA demonstrated
8 significant differences between the ultrasound speeds measured in two orthogonal
9 directions and among different regions.

10 **Fig. 8.** Ultrasound speed measured in the vertical slice throughout the full thickness of
11 AC. The presented data were the average of the ten specimens and the error bars
12 indicated the standard deviations. It was demonstrated that the ultrasound speed was
13 significantly dependent on the depth of AC.

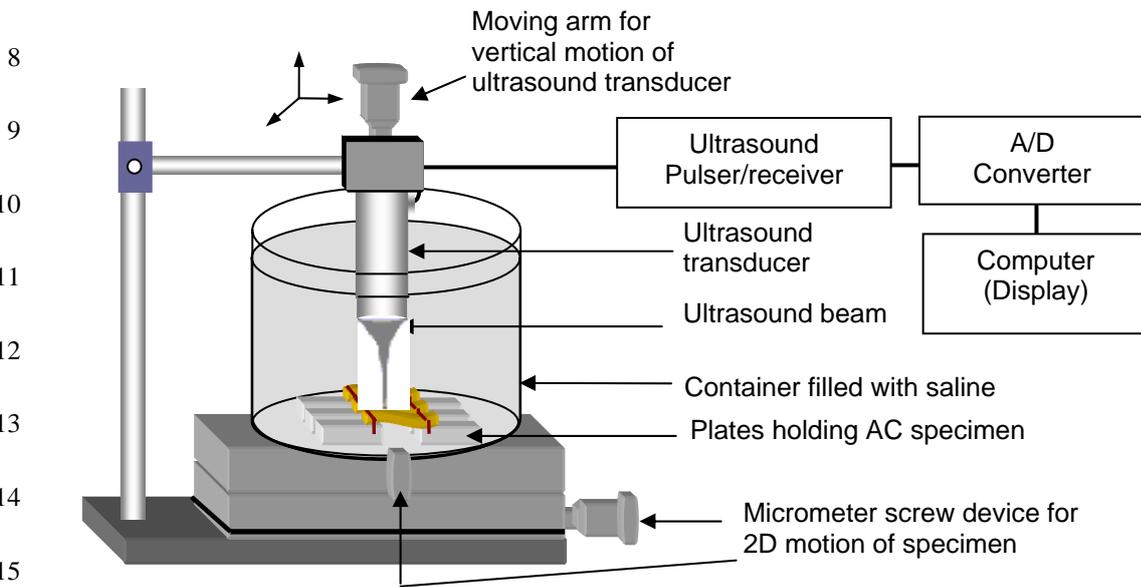
14 **Fig. 9.** The changes of (a) normalized AC thickness and (b) normalized sound speed
15 as a function of time after the specimen was detached from the bone. Ten full-
16 thickness slice specimens were prepared from 5 disk specimens, which were cored
17 from 5 different bovine patellae. The 2 slices from each disk specimens were tested in
18 two different orthogonal directions, respectively. No distinguished difference was
19 noted between the results measured in the two directions. Hence the results shown in
20 the figure were the combined data of all the 10 slices from the 5 patellae.

21

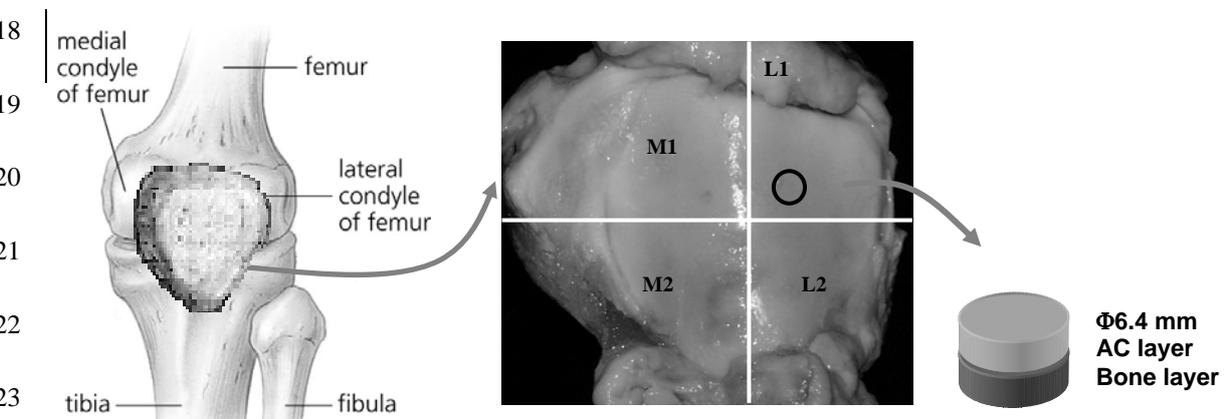
1 **FIGURES**



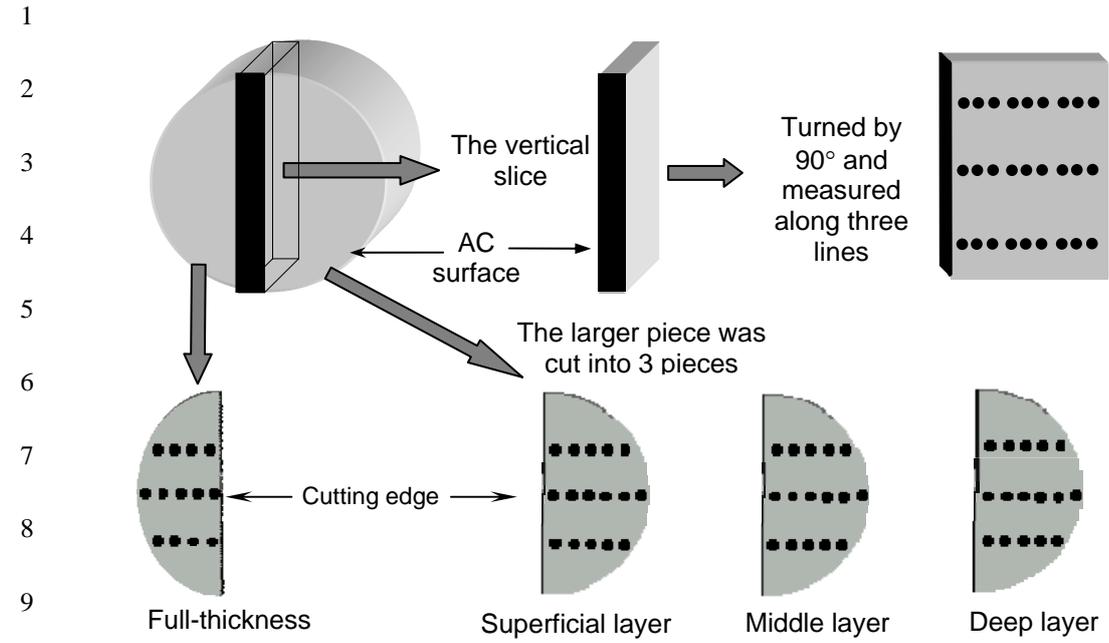
7 **Fig. 1**



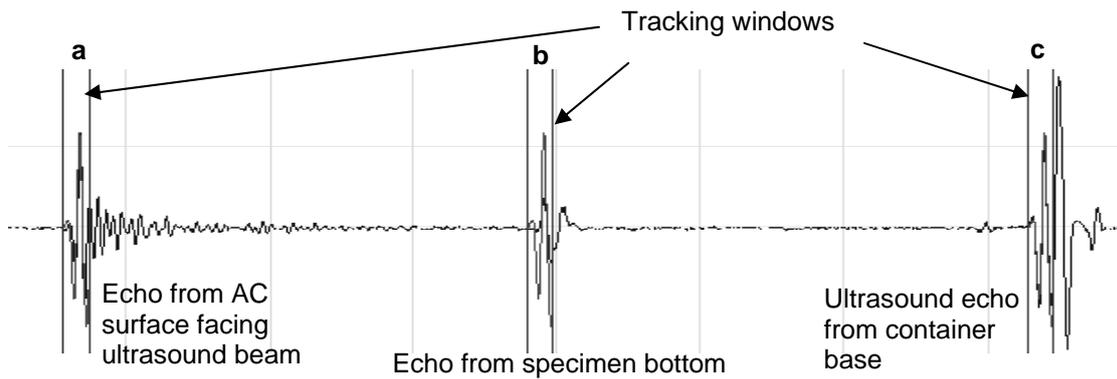
16 **Fig. 2**



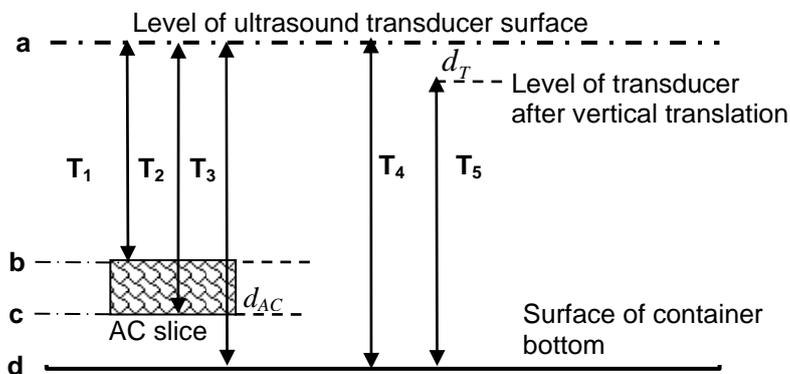
25 **Fig. 3**



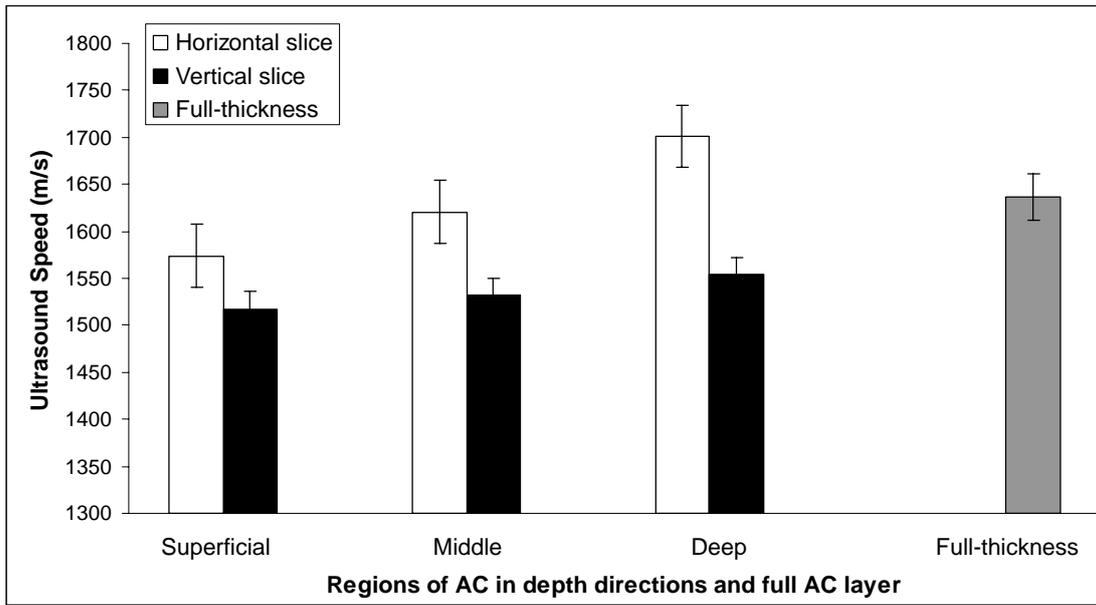
11 **Fig. 4**



18 **Fig. 5**



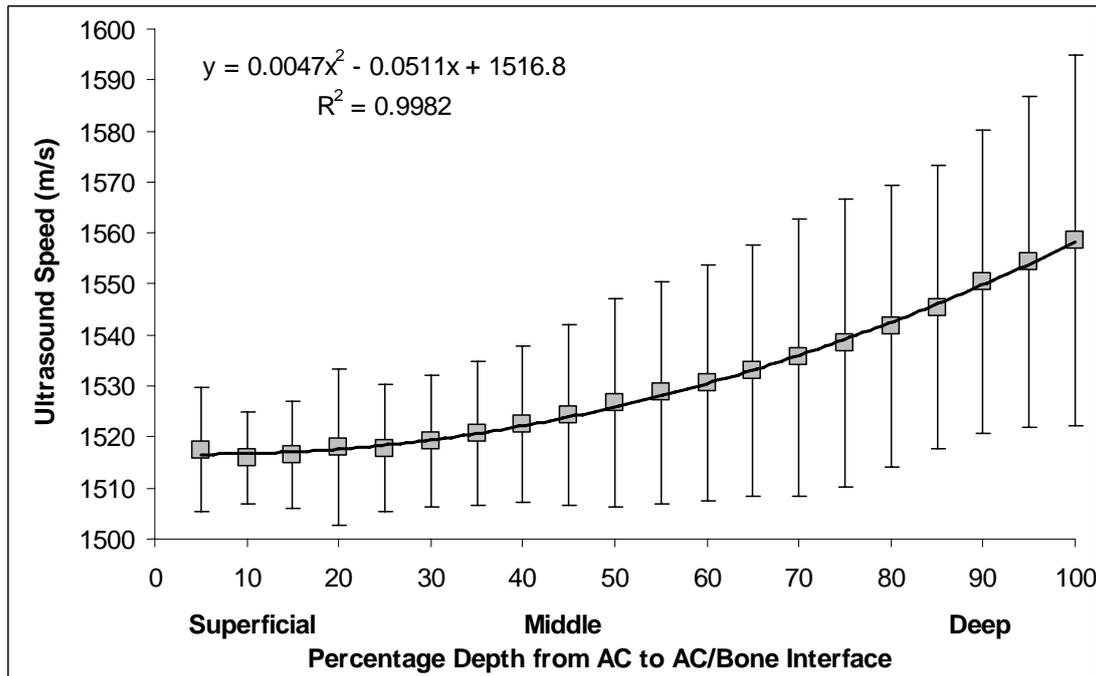
1 **Fig. 6**



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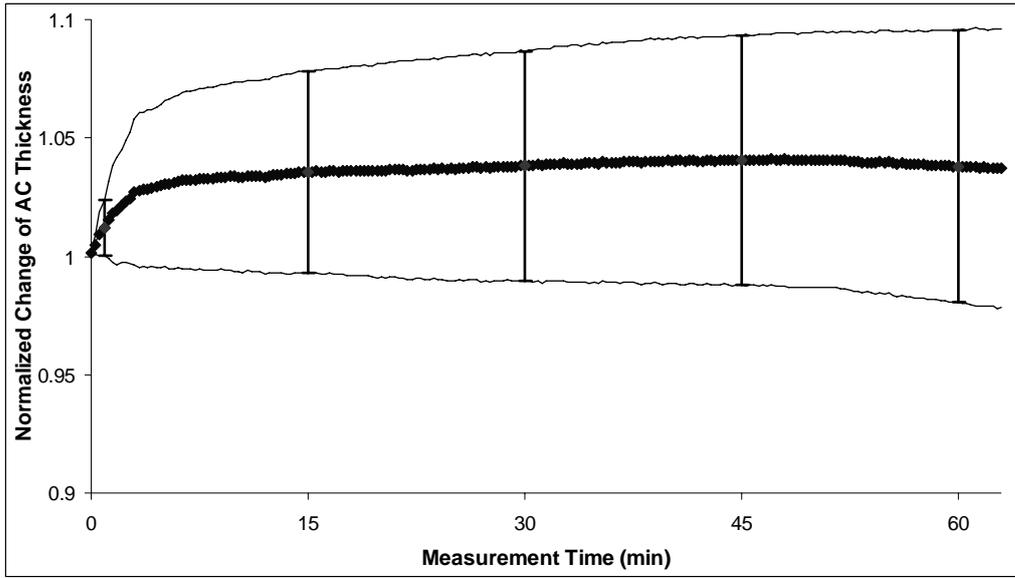
3 **Fig. 7**

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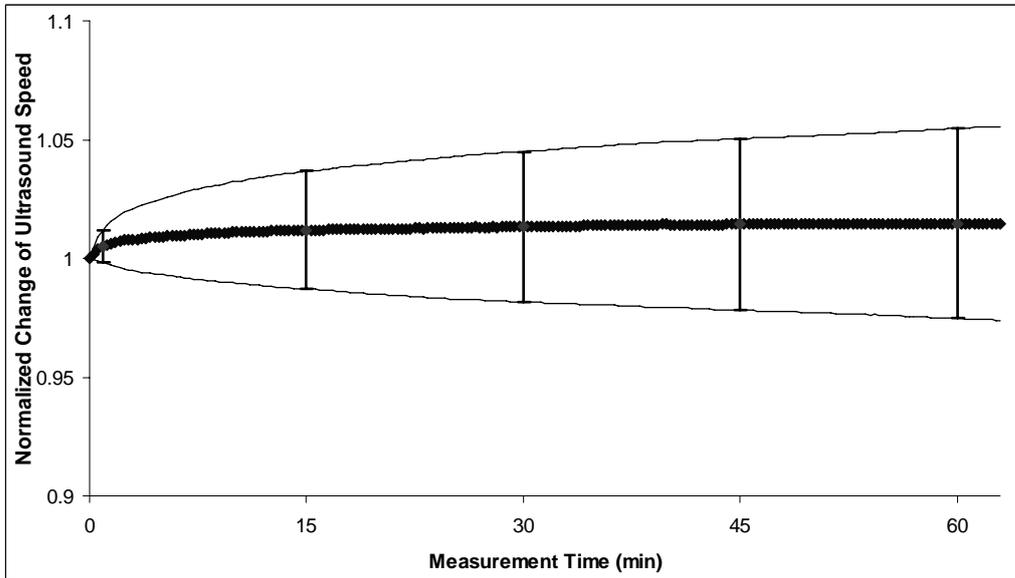


5

6 **Fig. 8**



(a)



(b)

Fig. 9

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1 **Table 1.** The mean normalized sound speed and thickness of AC slice specimens
2 measured at different time after they were detached from their subchondral bones and
3 installed on the specimen platform (corresponding to Fig. 9). Ten full-thickness slice
4 specimens were prepared from 5 disk specimens, which were cored from the
5 approximately same location of 5 different bovine patellae. The 2 slices from each
6 disk specimens were tested in two different orthogonal directions, respectively. The
7 results obtained in the two directions were combined together in the table, as no
8 significant differences were demonstrated due the change of the measurement
9 direction.

10

Time (min)	Normalized Speed		Normalized Thickness	
	Mean	SD	Mean	SD
1	1.005	0.007	1.012	0.012
15	1.012	0.025	1.036	0.043
30	1.013	0.032	1.038	0.049
45	1.014	0.036	1.041	0.053
60	1.014	0.040	1.038	0.058

11

12

1 **Table 2.** A summary of the sound speed of full-thickness AC reported in the literature.
 2 The information of the specimen type, ultrasound frequency, and testing temperature
 3 were included. In addition, the methods used for thickness measurement were also
 4 described.

Authors (Year)	AC Specimen	Ultrasound frequency	Testing temperature	Thickness measurement	Sound speed
Modest et al. (1989)	Human femoral head	7.5 MHz	Room temperature	Microscope and needle insertion	1760 m/s
Jurvelin et al. (1995)	Canine and bovine femoral and tibial condyles	10 MHz	Room temperature (22.5°C)	Microscope and needle insertion	1760 m/s (verified)
Agemura et al. (1990)	Bovine patella	100 MHz	Room temperature	Optical method	1617 ~ 1720 m/s
Myers et al. (1995)	Human femoral condyle	25 MHz	Room temperature	Microscope	1658±185 m/s
Yao et al. (1999)	Human hip and ankle joint	20 MHz	Room temperature	Needle insertion	1892±183 m/s
Toyraas et al. (1999)	Bovine patella	22 MHz	37°C	Needle insertion	1654±82 m/s
Joiner et al. (2001)	Bovine femoral condyle Human femoral condyle	30 MHz	37°C	Ultrasound propagation in saline with specimen in contact with the bottom	1666±16 m/s 1664±7 m/s
Suh et al. (2001)	Bovine patellar and femoral condyle	10 MHz	Room temperature	Microscope	1735±35 m/s
Toyraas et al. (2003)	Bovine femoral condyle, patella, patello femoral groove and talus joint	10.3 MHz	Room temperature (20°C)	Microscope	1627 m/s (1532 ~ 1754 m/s)
Present study	Bovine patella	50 MHz	Room temperature (19.5 ± 1°C)	Ultrasound propagation in saline with specimen lifted	1626±25 m/s

5