Real-Time Ultrasonic Assessment of Progressive Proteoglycan Depletion in Articular Cartilage

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Abstract

The loss of proteoglycan (PG) is regarded as one of the early signs of osteoarthritis (OA), thus observing the progress of PG loss would be useful for the early detection of OA. In this study, high-frequency ultrasound was used to monitor and analyze the trypsin-induced progressive degeneration in articular cartilage. Full-thickness cartilage-bone specimens (n = 10) prepared from normal bovine patellae were digested using 0.25% trypsin solution for different periods of time to evaluate the dynamic of the digestion process. The trypsin penetration front was observed in M-mode image, which was acquired using a nominal 50 MHz focused transducer. The transient speed of the digestion process was estimated from the image. The digestion fraction, which represents the ratio of the digestion depth to the total cartilage thickness, was estimated from ultrasound data and histology sections. With ultrasound, the digestion fraction observed in the 10 specimens ranged from 64 to 99%, and was correlated to that measured by histology ($R^2 \geq 0.63$, $p<0.05$). It was found that the digestion speed decreased nonlinearly with depth from 0.61 ± 0.16 μm/s (mean ± SD) in the superficial zone to 0.04 ± 0.02 μm/s in a region located at 70% of the cartilage thickness in depth. The relationship between the digestion depth and the exposure duration in trypsin could be described using a 3rd order polynomial function. The full-thickness of digested and undigested tissues was also measured using caliper, estimated from ultrasound data and histology sections, and compared. These findings indicate that ultrasound could provide useful information about the trypsin-induced progressive PG depletion in articular cartilage. Therefore, ultrasound represents a useful tool to evaluate the dynamic of models of OA *in vitro* in cartilage specimens in a research environment, and this would ultimately help the *in-vitro* examination of articular cartilage for research related to model of OA from the early stages of tissue degradation.

Keywords: Articular cartilage; Ultrasound; Proteoglycan depletion; Trypsin digestion; Osteoarthritis

INTRODUCTION

Articular cartilage is a low frictional, load-bearing soft tissue, which covers the articulating bony ends in diarthrodial joints. This tissue is a multi-phasic hydrated mixture mainly composed of 5–10% proteoglycan (PG), 10–20% collagen, and 60–80% water (Mow et al. 2005). It has been discovered that the aggregating PGs are bio-macromolecules negatively charged and enmeshed in the collagen matrix (Lai et al. 1991; Maroudas 1976). PGs are also involved in binding cations and water. Therefore, they play an important role in electrochemical mechanical properties of articular cartilage such as shear modulus (Zhu et al. 1993), compressive modulus (Qin et al. 2002; Zheng et al. 2001), swelling strain (Narmoneva et al. 1999; Wang and Zheng 2006) and swelling aggregate modulus (Flahiff et al. 2004; Narmoneva et al. 2002; Wang et al. 2007).

Osteoarthritis (OA) is considered as one of the most common joint diseases, which in its advanced stage is characterized by a partial or total loss of the cartilage tissue and the exposition of the bone across the joint. At a late stage, swelling and pain can lead to the loss of the joint functions, and consequently immobility. It was suggested that a macromolecular degradation happens at the early- and mid-stages of the cartilage degeneration and is catalyzed by proteolytic enzymes (Sandy 2003). The increase of tissue hydration, loss of PGs, and damage of collagen fibrils were regarded as the earliest signs of cartilage degeneration during OA (Armstrong and Mow 1982; Martini 2004; Sandy 2003; Torzilli et al. 1990). Since these early signs are not easy to detect, generally, articular cartilage has already suffered serious and irreversible damage when OA can be clinically diagnosed using X-ray. Therefore, detecting early signs of cartilage

degeneration, such as the loss of PGs, would allow an early diagnosis of the disease, and a timely treatment.

During the past decades, several methods and techniques have been developed and used for the evaluation of the cartilage degeneration. Histological assays are traditionally used for the assessment of cartilage degeneration by binding different stains, such as safranin O, toluidine blue, haematocylin and eosin (H&E), and alcian blue, to different compositions (Lyons et al. 2006). However, this method is time-consuming and invasive since it requires removing samples from the tissue using biopsy. Imaging techniques, such as magnetic resonance imaging (MRI) (Batiste et al. 2004; Raynauld et al. 2006) and ultrasonography (Yang et al. 2005), have recently been used in clinical practice providing non-invasive approaches to measure morphological damages in articular cartilage. The damages include irregularities at the cartilage-bone interface and the loss of the cartilage volume. High frequency ultrasound characterization of articular cartilage has been the subject of many recent investigations. These studies have reported on the suitability of ultrasound for the measurement of articular cartilage thickness (Adam et al. 1998; Joiner et al. 2001; Jurvelin et al. 1995; Laasanen et al. 2002; Lefebvre et al. 1998; Myers et al. 1995; Toyras et al. 2001; Yao and Seedhom 1999). Various acoustic parameters including the speed of sound (Agemura et al. 1990; Myers et al. 1995), attenuation (Senzig et al. 1992; Toyras et al. 1999), echo pattern (Kim et al. 1995; Myers et al. 1995; Saied et al. 1997), and reflection and scattering coefficients (Adler et al. 1992; Cherin et al. 1998, 2001; Hattori et al. 2003, 2005; Laasanen et al. 2002; Nieminen et al. 2002; Pellaumail et al. 2002; Toyras et al. 1999) have been used for the characterization

of articular cartilage in healthy and osteoarthritic conditions. 3D ultrasound measurement of articular cartilage has also been reported (Lefebvre et al. 1998). A miniaturized A-mode ultrasound probe has been developed for arthroscopic use (Hattori et al. 2005; Laasanen et al. 2002). In addition, ultrasound has been combined with indentation and compression for the measurement of tissue elasticity (Fortin et al. 2003; Laasanen et al. 2002; Saarakkala et al. 2003; Toyras et al. 1999; Zheng and Mak 1996; Zheng et al. 2001, 2002, 2004a, 2005). However, experiments reported in most of these previous studies were performed *in vitro* and acoustic and mechanical properties were measured at only two time points, before and after cartilage degeneration.

Recently, the dynamic of trypsin penetration during digestion into the cartilage has been investigated using ultrasound (Nieminen et al. 2002; Zheng et al. 2004b). In the early stage of OA, PGs are continuously being broken down and PG fragments are released from the matrix leading to the net loss of PGs. Similarly, trypsin digestion can induce a similar cleavage of PG aggregates. Therefore digestion of cartilage using this agent has been widely used as a model of OA. Traditionally, the effects of trypsin in cartilage have been evaluated by histology at discrete time points only. Since variations in the cartilage thickness and in the distributions of PGs and chondrocytes are commonly observed between different specimens (Moody et al. 2006), it is difficult to control the digestion process by trypsin using histological techniques. On the other hand, it has been well documented that the loss of PGs causes a significant reduction of the tissue elasticity, which is directly related to the acoustic impedance. It has been shown that a rupture of impedance between digested and undigested tissue leads to the detection of an ultrasound

echo at the interface between these tissues. The motion of this echo toward the bone, as trypsin penetrates deeper in the cartilage, has been observed in M-mode images (Nieminen et al. 2002; Zheng et al. 2004b).

In this study, we investigate this ultrasound approach for tracking in real-time the penetration of trypsin and measuring the transient speed of digestion. To validate this method, measurements performed with ultrasound are compared with those obtained using histology. Our results might provide useful information about the dynamic of the digestion by trypsin and therefore some insight on the early stages of OA. They might also provide a validation of the ultrasound approach for a real time evaluation of models of OA.

MATERIALS AND METHODS

Specimen preparation

Fresh mature bovine patellae (n = 10) without apparent lesions were obtained from a local butcher shop within 6 hours of slaughter and stored at -20°C until further preparation. One cartilage-bone plug was obtained from the upper-medial area of each patella using a metallic punch with a diameter of 6.35 mm (Wang and Zheng 2006). The cartilage-bone plugs were wrapped in wet gauze soaked with physiological saline and stored at -20°C until ultrasound examination. Before examination, each plug was cut into two parts (1/3 and 2/3) (Fig. 1a). The 2/3 portion samples were exposed to trypsin digestion and monitored by ultrasound simultaneously. Both trypsin-treated (2/3 portion) and control (1/3 protion) samples were assessed using histology.

Ultrasound examination and signal processing

Before ultrasound examination, each sample was first thawed for three hours in physiological saline solution (0.15 M NaCl) at room temperature (21±1°C). Then, the sample was fixed using the rubber gel (Blu-Tack, Thomastown, Australia) to the bottom of the container. The outer ring of the surface of the cartilage disc, approximately 0.6 mm in thickness, was gently covered by the rubber gel to ensure a trypsin penetration into the cartilage sample from the surface top layer toward the cartilage-bone interface (Fig. 1). The sample was then submerged in 0.15 M saline solution for another one hour. Then, the saline solution was rapidly removed from the container using an injection syringe and replaced with a trypsin solution (0.25%, Cat. No. 25200-056, GiBCO, Invitrogen Inc., Burlington, ON, Canada). The fluid replacement was performed in approximately 30 s.

Trypsin digestion in the sample was monitored using an ultrasound measurement system developed in our laboratory (Fig. 1b) (Wang and Zheng 2006). An ultrasound pulser/receiver (Model 5601A, Panametrics, Waltham, MA, USA) was used to drive a nominal 50 MHz focused ultrasound transducer (Model PI50-2, Panametrics, Waltham, MA, USA). This transducer had a focal length of 12.7 mm, a diameter of 6.35 mm, a -6dB beamwidth of 0.1 mm and a -6dB focal zone depth of 0.95 mm. The focal zone of the transducer was positioned in the centre of the sample using a 3D translating stage (Model R301MMX/2201MMXY, Ball Slide Positioning Stages, Del-Tron Precision Inc., Bethel, CT, USA). Ultrasound echoes reflected or backscattered from internal structures in the cartilage sample were collected by the transducer, amplified by the receiver, and

digitized by an 8-bit A/D converter at a sampling rate of 500 MHz (CompuScope 8500PCI, Gage, ON, Canada). Digitized signals were displayed on the computer monitor in real-time and simultaneously saved on the hard drive for offline data analysis. A custom-designed software developed in our laboratory for the ultrasound measurement of motion and elasticity (Zheng et al. 2001, 2004b) was used to control data collection and display.

During the progressive digestion of cartilage by trypsin, M-mode data were collected at a pulse repetition frequency (PRF) of 0.6 Hz. M-mode images are particularly suitable for monitoring of movements of tissue interfaces, and have been commonly used to study the motion of heart walls. Experiments were carried out at room temperature (21±1°C). As the trypsin penetrated into the cartilage tissue and broke down PG aggregates, an echo appeared at the interface between digested and undigested tissues. With time, this echo was detected deeper in the tissue, and gave rise to the inclined trace observed in M-mode images (Fig. 2). Markers were manually positioned along the trace in the M-mode images at different depths corresponding to the location of the digestion front at different time points. From the locations of these markers, our software automatically generated by interpolation the curve representing the depth of this front as a function of time. The positioning of the markers was repeated six times for each specimen to obtain six different interpolation curves, which were averaged together. From this averaged curve, were extracted both the transient speed of trypsin penetration and the digestion fraction. The digestion fraction was defined as the ratio of the digestion depth to the total cartilage thickness. For the comparison between some ultrasonic and histological measurements,

the digestion fraction was preferred to the absolute digestion depth, because it is not affected by variations of the ultrasound speed between samples or a potential shrinkage induced by the histological process.

In this study, the full thickness of the cartilage layer (h) was calculated by the following equation.

$$h = c_{AC} \times T_{AC} / 2$$

where T_{AC} is the time of flight that the ultrasound echoes travel through the cartilage tissue. The ultrasound speed in articular cartilage c_{AC} was calculated using a non-contact ultrasonic method (Patil et al. 2004). The average sound speed in the full-thickness cartilage layer soaked in 0.15 M saline was 1675 m/s for the normal specimens. Since the echoes reflected from the cartilage-bone interface shifted slightly during the digestion (Fig. 2), indicating that the trypsin digestion generated a corresponding slight change in the ultrasound speed in the tissue, a speed of 1668 m/s was used for the degenerated tissue (Nieminen et al. 2002).

The trypsin digestion was stopped by removing the enzyme solution when the front echoes reached a depth of at least 60% of the total cartilage thickness. The specimens were immediately washed with physiological saline and stored in a refrigerator until the histological analysis was performed.

Histology

All control and trypsin digested samples were fixed in a 10% buffered formalin and then quickly decalcified in a 10% EDTA solution, using an ultrasound method reported by Guo et al. (2005), until the bone tissue could easily be cut with a scalpel. Paraffin sections, 4μm, were prepared using a rotatory microtome (Lecia RM-2135, Cambrigde, UK). During section trimming, care was taken to obtain sections proximal to the center part of the specimen, where the region examined by ultrasound was located. Then, the deparaffined sections were stained with safranin O (Cat. No. F7258, SiGMA, MO, USA) and contra-stained with fast green (Cat. No. S-2255, SiGMA, MO, USA). The sections were examined by an imaging system including a microscope (Model FN-S2N, Nikon, Japan) and a digital camera (DXM 1200X, Nikon, Japan). In optical micrographs, red color stained by safranin O indicated the presence of PGs (Leung et al. 1999; Qin et al. 2002). The depth of the trypsin digested zone estimated by measuring the depth of the fast green-stained zone in the histological image was normalized by the full cartilage thickness. For each specimen, the digestion fraction was estimated from three histological sections, averaged and compared to the ultrasound measurement.

RESULTS

The digestion depth is represented as a function of time in Fig. 3a for all tested specimens. The average digestion depths at 1 hr, 2 hr, and 3 hr were 0.76±0.10 mm, 1.02±0.12 mm, and 1.23±0.14 mm, respectively. Fig. 3b shows the relationship between the digestion depth and the required trypsin treatment time. The error bars are the standard deviations over the 10 specimens. A 3rd order polynomial function was used to fit this relationship

(R^2 = 0.9999). For a cartilage specimen with a given thickness, the approximate digestion time can be calculated from this relationship.

A comparison of ultrasound data and histological sections in terms of digestion depth is shown in Fig. 4 for two typical specimens. Fig. 4a, 4b and 4c represent respectively the histological section of a specimen of undigested cartilage (control sample), the M-mode data showing the complete digestion process from the cartilage surface to the bone, and the histological section of the completely digested sample (specimen 8). Fig. 4d, 4e and 4f represent the same types of images for an incompletely digested specimen (specimen 1). The results of ultrasound and histological measurements of the digestion fraction are shown in Fig. 5. With ultrasound, the final digestion fraction ranged from 64% to 99% of the sample full-thickness. A correlation was found between the two types of measurement ($R^2 = 0.63$, $p < 0.05$), although a significant bias (0.64) was found between the ultrasound and histological measurements. For 7 out 10 specimens, the digestion fraction measured by histological sections was larger than that measured with ultrasound. Also, it was found that ultrasound measurements correlated better with histology for completely digested samples than for samples that were only partially digested. The potential reasons are discussed in the next section. Therefore, the cartilage full-thickness measured by ultrasound was correlated with both histological and digital caliper measurements for both normal cartilage and digested cartilage (slope ≈ 1, R^2 ≈ 0.9) (Fig. 6). A statistical analysis (paired-samples t-test) of the full-cartilage thickness obtained using any of the three measurement methods, showed that there was no significant

difference between digested and undigested samples ($p > 0.9$). The average full-thickness of the control samples measured by ultrasound, digital caliper and histology was 1.76±0.46 mm, 1.74±0.41 mm, and 1.82±0.47 mm, respectively; that of the digested samples was 1.78±0.48 mm, 1.72±0.44 mm, and 1.82±0.42 mm, respectively.

The transient speed of the digestion front echo was calculated at different time points for the first three hours. The averaged transient speed over all samples is shown as a function of time in Fig. 7a. Fig. 7b shows the digestion front speed as a function of depth in the tissue. It can be seen from Fig. 7 that the digestion speed decreased with increasing tissue depth. The digestion speed was found to be faster in the superficial layer of the cartilage samples (0.61 ± 0.16 μm/s, mean ± SD) than in deeper layers (0.04 ± 0.02 μm/s at 70% of the full thickness).

DISCUSSION

A relatively good correlation was found between ultrasound and histology in the measurements of the digestion fraction ($R^2 = 0.63$, $p < 0.05$). However, for partially digested samples, the digestion fraction measured in histology was higher than that estimated using ultrasound. This difference could potentially be explained by a number of factors affecting these two techniques. On the histology side, these factors might include tissue shrinkage due to the fixation process, limitation in the staining by safranin O, and a potential residual digestion by trypsin between the end of the ultrasound experiments and the histological process. On the ultrasound side, the only factor is a potential difference in the speed of sound between digested and undigested tissues.

Although, tissue shrinkages or deformations due to fixation processes have been reported in literature, the effects of the fixation by formalin, which has been used in our study, are minimal (Luna 1992). In this study we made the assumption that if shrinkage occurs, digested and undigested tissues will be affected in the same proportion. This hypothesis seemed to be verified in our experiments, since no significant difference in total thickness was found between partially digested samples and their controls. Therefore, shrinkage should not affect the digestion fraction, which represents the depth of PGs digestion normalized by the total tissue thickness.

Staining by safranin O, on the other hand, might affect our histological findings. Indeed, a study published by Camplejohn and Allard (1988) showed that some PGs (chondroitin sulphate and keratan sulphate) were still present in regions of the cartilage not stained by safranin O. Therefore, this would lead, in our experiments, to an overestimation of the depth of digestion, since it was estimated by measuring the depth of the region unstained by safranin O. Consequently, the digestion fraction would be overestimated.

Some residual digestion by trypsin might have occurred as well between the time the trypsin solution was removed from the bath and the time the tissues were fixed for histology. In future experiments, this could be prevented by using enzyme inhibitors to stop the digestion by trypsin (Nieminen et al. 2002; Qin et al. 2002). This would require investigating the dynamic of the inhibition process, which could be potentially done using our ultrasound technique.

Difference in the speed of sound between digested and undigested tissues might affect digestion fraction measured in ultrasound. Previous studies have reported that the ultrasound speed in PG-depleted cartilages is lower than in normal tissues (Joiner et al. 2001; Nieminen et al. 2002; Toyras et al. 1999). However, if we assume a constant speed of sound both in digested and undigested tissues in our experiments, the thickness of PG-depleted specimens would be overestimated. Furthermore, no significant difference was found in cartilage thickness between the PG-depleted specimens and their respective control using caliper, ultrasound and histology. Since a good correlation was found in the measurement of the total cartilage thickness between histology and ultrasound, we can also conclude that the choice of a speed sound of 1668 for the digested samples was probably accurate. From this analysis, we can infer that the difference in digestion fraction between histology and ultrasound is probably due to an inaccurate estimation of the digestion depth of histology.

The speed of digestion was found to decrease as increasing of tissue depth. This tendency is related to the distribution of the PG content, which has been reported to increase as a function of the tissue depth in normal articular cartilage (Mow and Guo, 2002; Wang et al., 2002). This implies that more trypsin is needed to digest the PGs deep in the tissue than at its superficial zone. This could slow down the trypsin penetration and thus the digestion process in the deeper region of the tissue. These results are consistent with results obtained by Moody et al. (2006), who showed that, for a given time of exposure and a given concentration of trypsin, the dynamic of PGs digested by this agent depends on the concentration of PGs at different depths in the tissue. In addition, this study

provides evidence of the variations in digestion by trypsin between different samples, as it is also reported in Moody's study. As shown in Fig. 3, sample 1 and sample 8, which are of equivalent thickness, behaved differently under exposure to trypsin. The dynamic of the diffusion of trypsin from the surface to the deepest region in the cartilage definitely requires further investigations.

In summary, our experiments demonstrated the feasibility of monitoring the digestion of PGs in real-time using an ultrasonic technique. Our results suggest that this technique might be better suited than histology to estimate the depth of digestion by trypsin for two reasons. First, this technique can evaluate the dynamic of the digestion in real-time. Secondly, the echo reflected at the interface between digested and undigested tissue represents a real marker of a change in local mechanical properties of the tissue, which are linked to the content in PGs in the tissue. Our results also provide information about the progressive depletion of PGs from the cartilage induced by trypsin. It was found that the digestion depth could be described as a 3^{rd} order polynomial function of the exposure time to trypsin. This ultrasound method could be used for other *in-vitro* models of OA based on PG degeneration.

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Figure Captions

Fig. 1 (a) The cartilage-bone plug was cut into two parts (1/3 and 2/3). (b) Schematic of the ultrasound experimental setup. Samples were fixed at the bottom of the container filled with saline or trypsin solution.

Fig. 2 Typical M-mode image showing the progression of the digestion induced by trypsin. Markers along the digestion front are indicating some of the positions of the front used for the calculation of the interpolation. RF ultrasound echoes (a, b and c) were extracted from the M-mode image. The echoes from the digestion front, which are circled in the RF signals, are pointed by black arrows in M-mode image.

Fig. 3 (a) The transient digestion depth as a function of time for the 10 specimens. (b) Nonlinear relationship between the digestion depth and the digestion time.

Fig. 4 Comparison of M-mode images with histological sections for a completely digested cartilage (a,b,c) and an incompletely digested cartilage (d,e,f). (a) and (d) are histological sections of the control undigested samples; (b) and (e) are M-mode images showing the digestion front dynamic; (c) and (f) are histological sections from the digested samples. Black triangle indicates the cartilage surface. Dashed line in (c, f) indicates the interface between safranin O and fast green stained regions. The scale is 200 μm.

Fig. 5 Correlation between the digestion fractions measured using histology and ultrasound.

Fig. 6 Correlation of cartilage full-thickness measured by: (a) digital caliper versus ultrasound; (b) histology versus ultrasound.

Fig. 7 (a) Averaged digestion speed as a function of time. (b) Averaged digestion speed as a function of depth. Error bars represent the standard deviations over 10 specimens.

Figure 1
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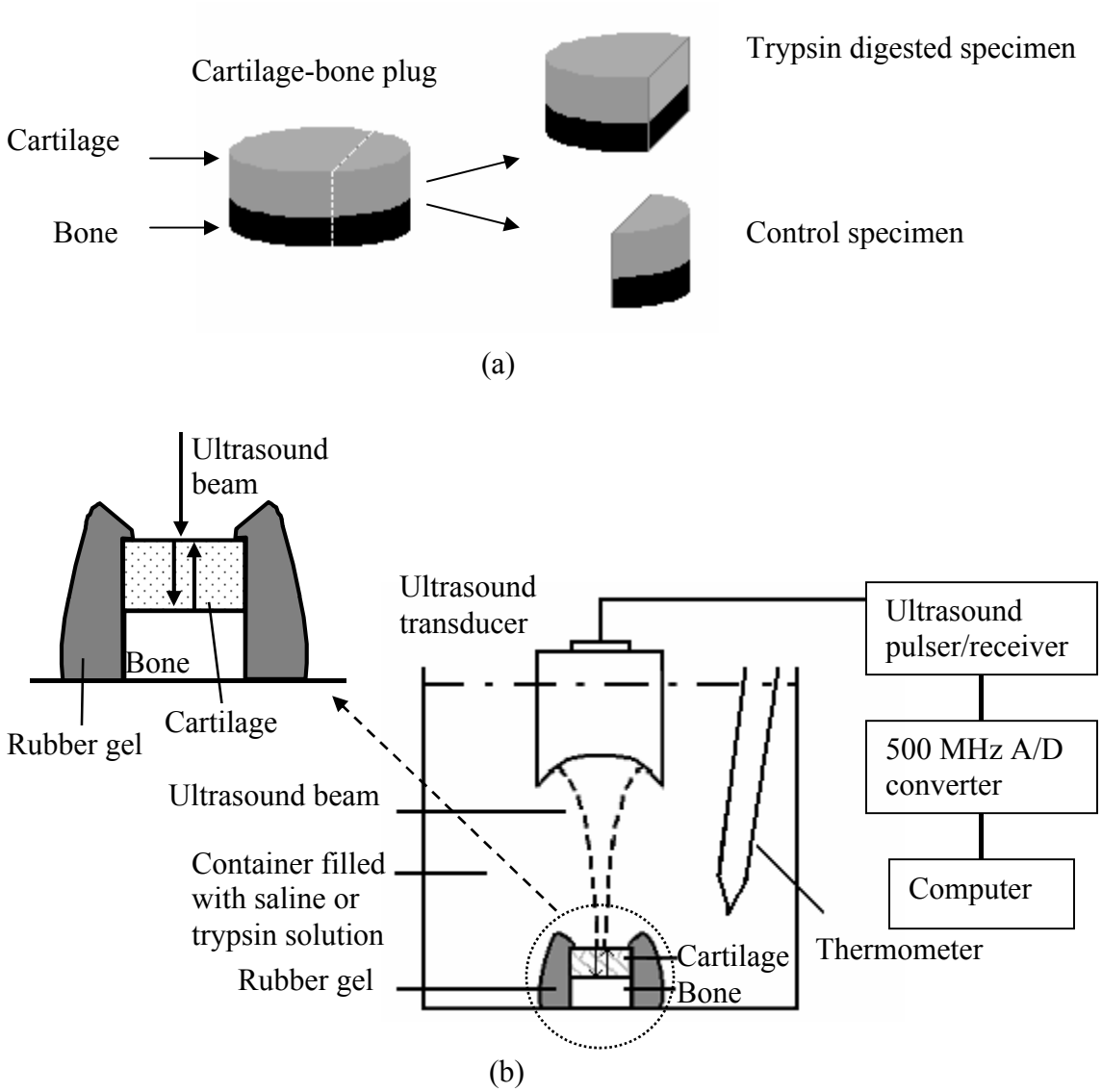

Fig. 1

Figure 2
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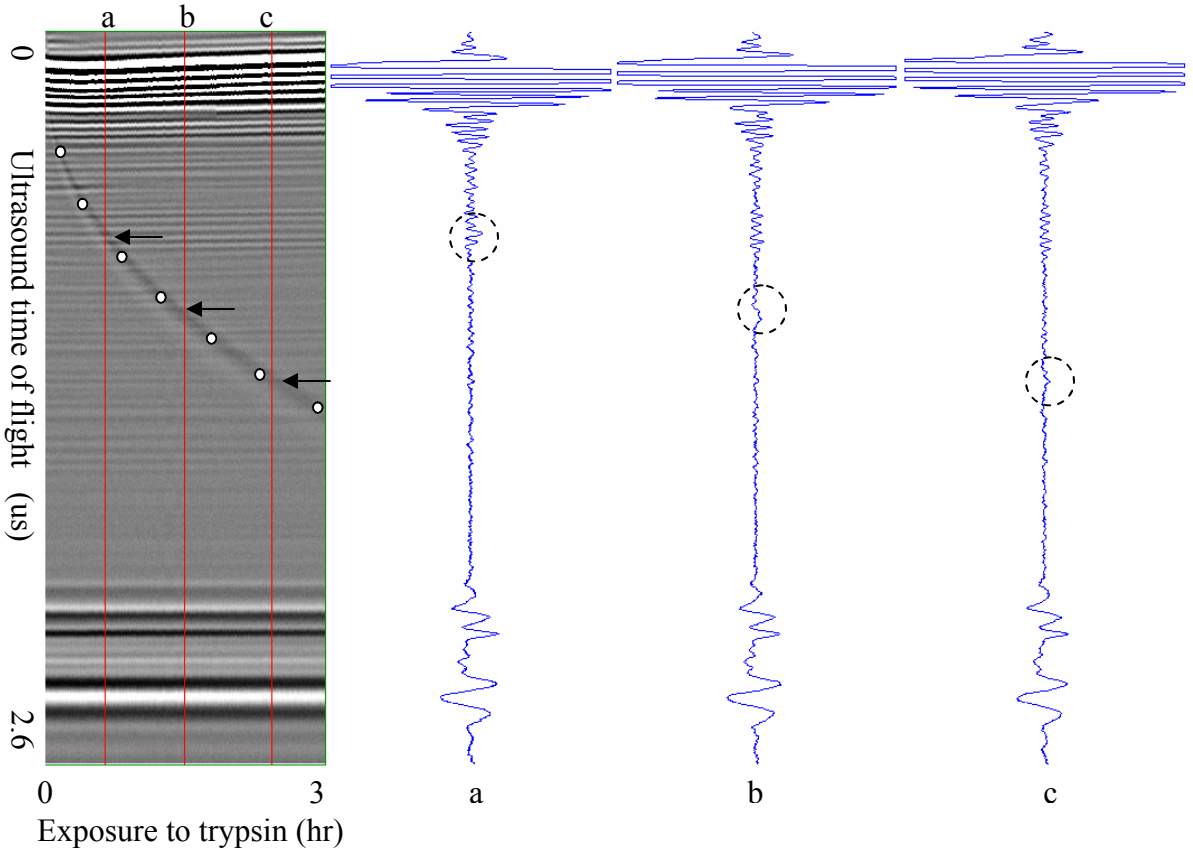

Fig. 2

Figure 3
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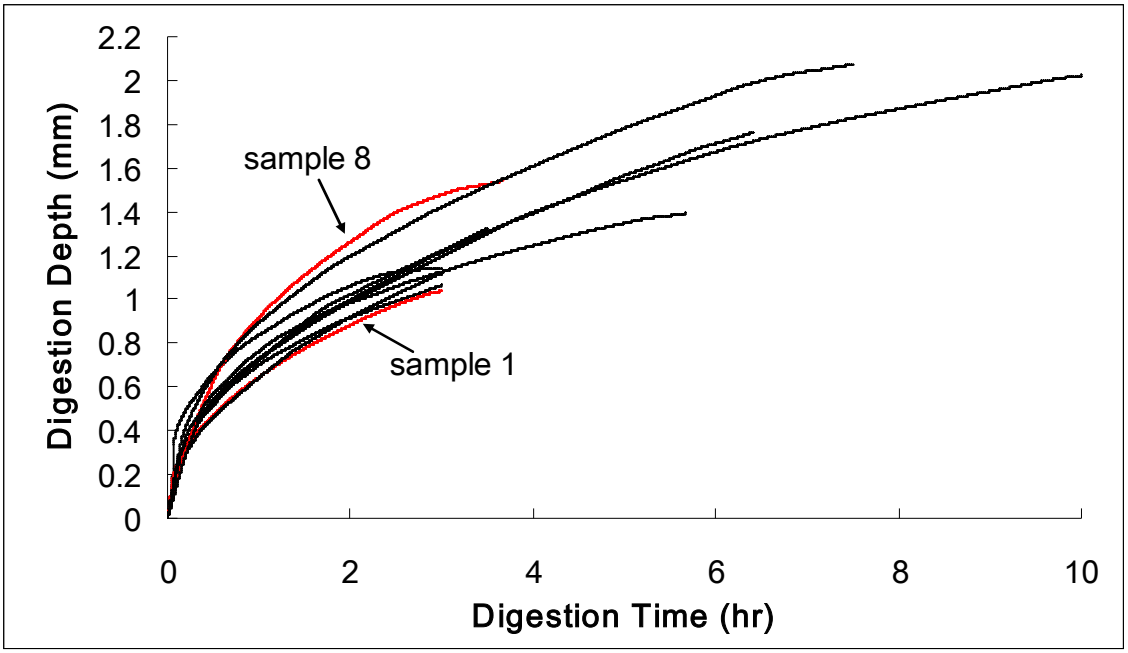

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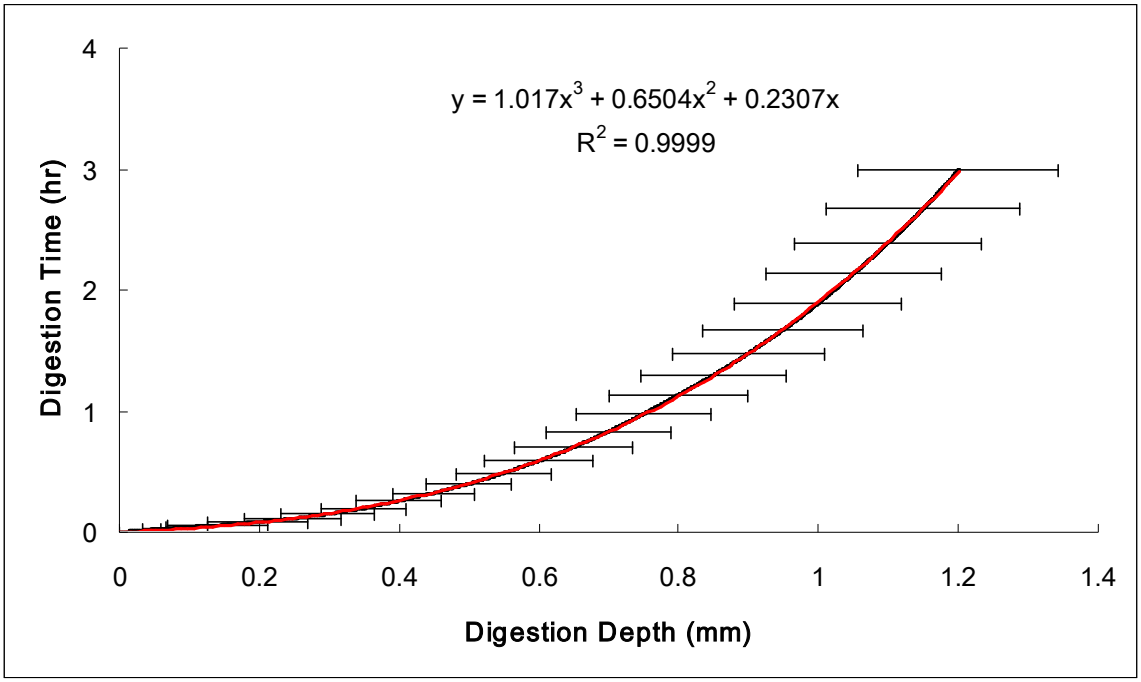

(b)

Fig. 3

Figure 4

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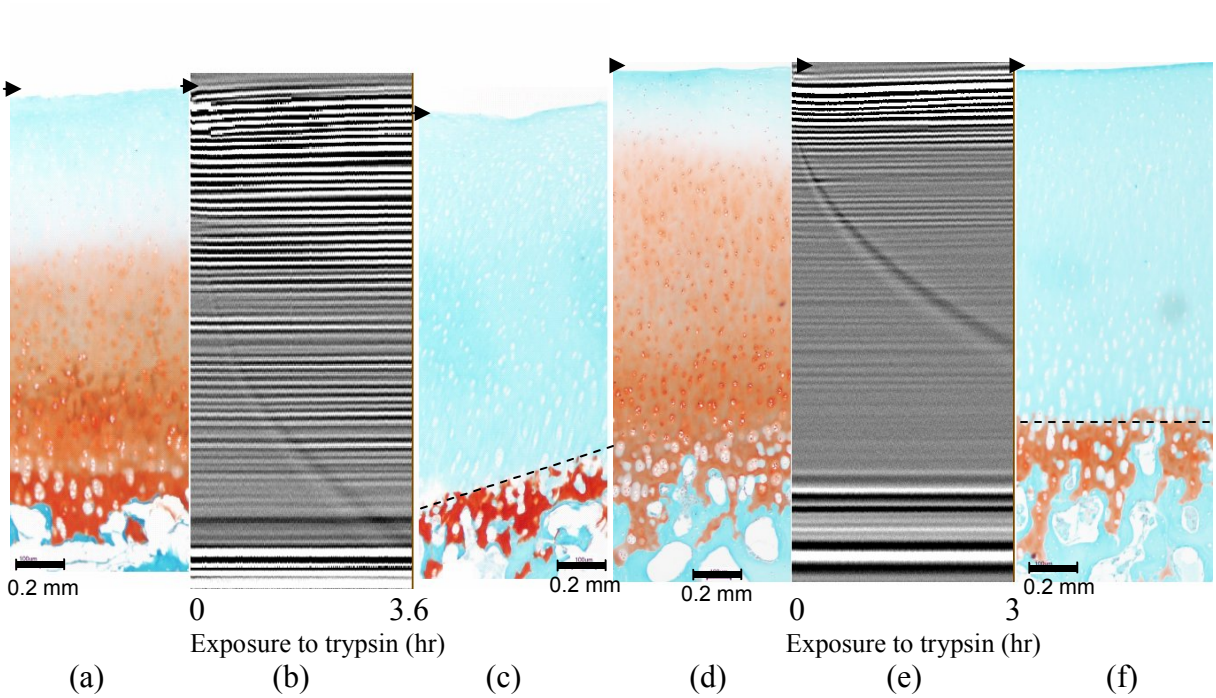

Fig. 4

Figure 5

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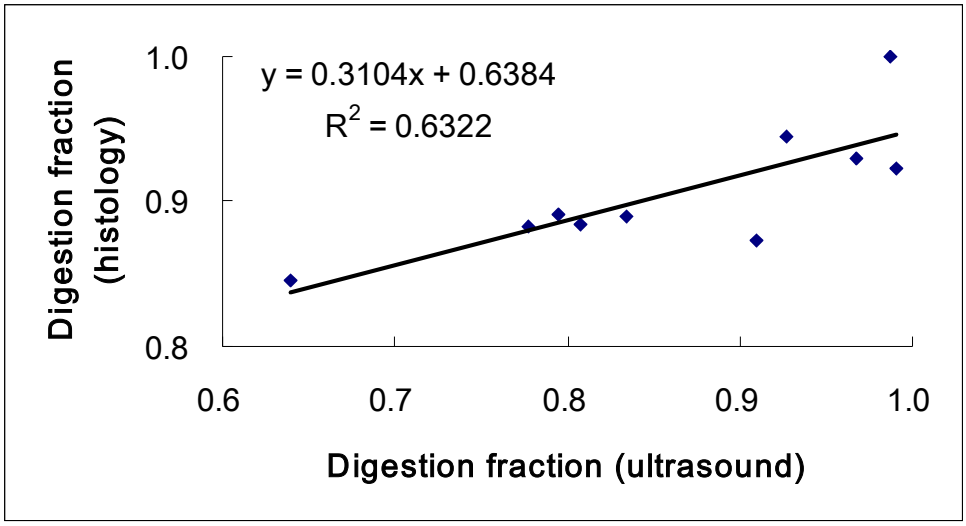

Fig. 5

Figure 6
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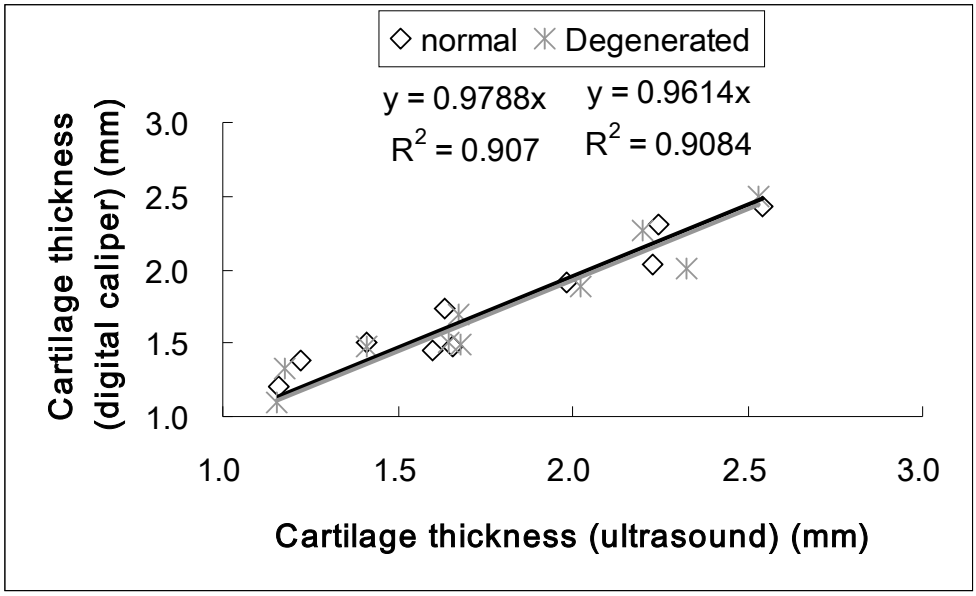

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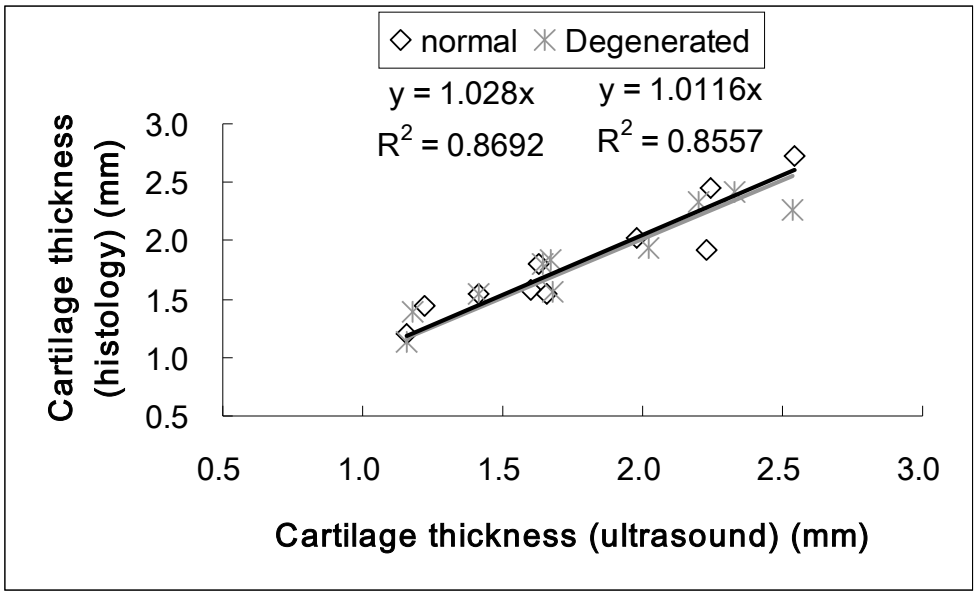

(b)

Fig. 6

Figure 7
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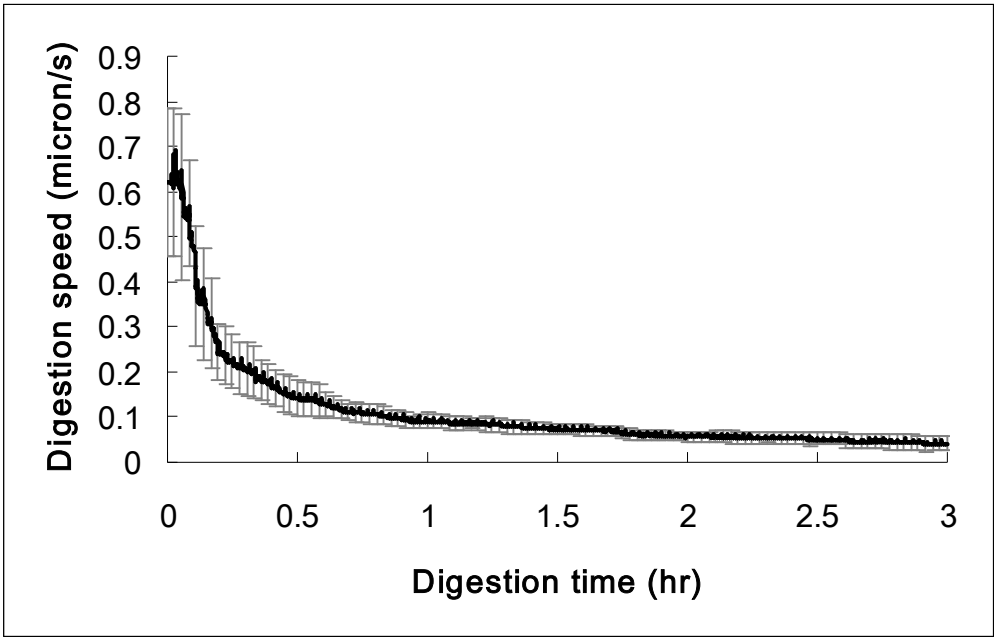

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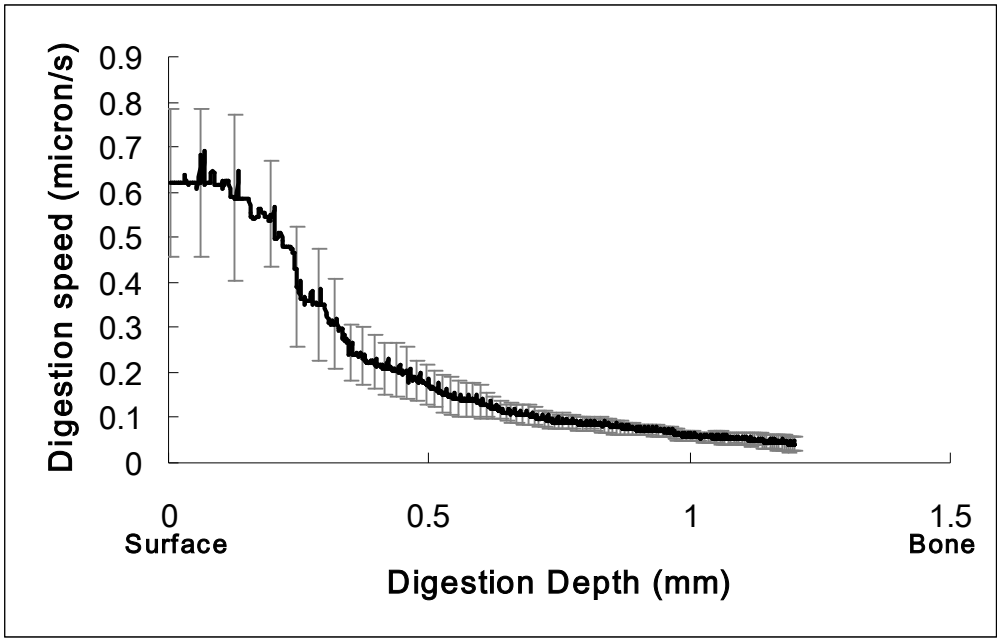

(b)

Fig. 7