METHODS ARTICLE

Rapid Detection of Shear-Induced Damage in Tissue-Engineered Cartilage Using Ultrasound

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Previous investigations have shown that tissue-engineered articular cartilage can be damaged under a combination of compression and sliding shear. In these cases, damage was identified in histological sections after a test was completed. This approach is limited, in that it does not identify when damage occurred. This especially limits the utility of an assay for evaluating damage when comparing modifications to a tissue-engineering protocol. In this investigation, the feasibility of using ultrasound (US) to detect damage as it occurs was investigated. US signals were acquired before, during, and after sliding shear, as were stereomicroscope images of the cartilage surface. Histology was used as the standard for showing if a sample was damaged. We showed that US reflections from the surface of the cartilage were attenuated due to roughening following sliding shear. Furthermore, it was shown that by scanning the transducer across a sample, surface roughness and erosion following sliding shear could be identified. Internal delamination could be identified by the appearance of new echoes between those from the front and back of the sample. Thus, it is feasible to detect damage in engineered cartilage using US.

Keywords: tissue failure identification, acoustic analysis, nondestructive testing, ultrasonography, tissue engineering, articular cartilage

Impact Statement

For tissue-engineered cartilage (TEC) to be a viable treatment for severe joint degradation, it must be able to function under cyclic compression and shear. Mechanical failure of a TEC implant is a serious problem. Using existing validation protocols, it is, in general, not known how many cycles the sample survived. This is critical information. We thus rely on histology, a destructive endpoint assay that can take weeks. We developed a rapid testing protocol to validate TEC before implantation. Ultrasound can, in near real-time and nondestructively, identify test-induced damage in TEC. This is an important step toward making better cartilage.

Introduction

TISSUE-ENGINEERED CARTILAGE (TEC) is emerging as a biological means for repairing degraded articular surfaces. However, if TEC is to be a viable alternative to current treatments for severe degradation such as total joint replacement, it must be able to perform the same functions as native tissue. These necessary functions are the ability to resist compression and shear forces. Unfortunately, experience to date has shown that TEC is not up to the task.^{1–6} Mechanical failure of a cartilage implant results in the failure of the repair procedure at best, and, at worst, might

cause worsening damage to remaining healthy joint structures. This is a serious problem, and it would thus be useful to have a testing protocol which could validate TEC before implantation, to avoid such complications. This investigation is focused on a common mode of TEC failure, namely damage under compression and sliding shear—that is, conditions under which the joint normally operates. Our group has shown experimentally that unlike in, for example, static compression tests, TEC can be severely damaged under these combined dynamic conditions.^{4–6} For this and previous investigations, damage is categorized as surface erosion and/or delamination, although we have also reported

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internal damage without delamination in TEC under static shear.⁷ Seeing damage of this nature *in vitro* would lead us to predict that the type of TEC being tested would fail *in vivo*, and that the tissue-engineered (TE) protocol needs further refinement. Currently, identifying the damage is dependent on histological examination of the tissue after the test is completed. Two problems with this approach are, first, that, even with a dedicated histologist on hand, it can take at least hours and usually weeks to get the results, and the histological assay is obviously a destructive endpoint assay.⁴⁻⁷ Second, unless the sample is visibly destroyed during a test, we do not know, even approximately, after how many cycles the damage occurred.

This investigation is part of a series of studies in our laboratory into the use of ultrasound (US) to evaluate TEC.⁸⁻¹⁰ In this study, we sought to test the feasibility of using US as a means for identifying damage in TEC subjected to compression and sliding shear nondestructively, in near real-time during the mechanical test. This would allow us to identify almost immediately when (i.e., after how many loading cycles) damage occurred.

Because of the considerable monetary costs, data acquisition and data processing associated with developing a device with a transducer array capable of periodically interrogating an entire sample during a damage test, we felt it was necessary to first demonstrate the general feasibility of the approach. In this article, we only attempt to determine whether different degrees of shear-induced damage observed by histology identify changes in US signal patterns. To this end, we first studied whether sliding shear damage could be detected using our existing sliding shear^{6,11} and US devices.⁸⁻¹⁰ Based on favorable results, we then constructed an integrated device combining both functions. The latter is capable of performing high-resolution line scans of the sample.

Materials and Methods

Damage to either scaffold-free TEC grown in the laboratory of one of the investigators (J.E.D.) or bovine calf knee articular cartilage was evaluated. The overall testing protocol (Fig. 1) consisted of recording US signals and

FIG. 1. Flowchart of the experiments in this article. *Left column* was done using the sliding shear device described previously and a separate US measurement system. *Right column* used a newer integrated device described in Figure 4. Other than ease-of-use, there are no fundamental differences between the two devices with respect to sliding shear loading. US, ultrasound.

stereomicroscope images of the surface of each sample before, during, and after compression and sliding shear. Two generations of the sliding shear device were used in these studies (see section “Sliding shear”). Following shear and US evaluation, tissue samples were processed for Toluidine blue and hematoxylin and eosin (H&E) histology.

Tissue sample preparation

For native bovine cartilage, samples were harvested from a fresh normal bovine calf femoral condyle obtained from a local butcher shop. Five osteochondral samples were cored from the fresh whole bone using a 6 mm diameter coring tool. The cores were then cut across their diameter to make semicylindrical samples and frozen at -20°C until use.

For TEC, similar to previous studies, rabbit auricular cartilage was dissected and sequentially digested with testicular hyaluronidase (660 U/mL in Dulbecco’s modified Eagle’s medium-low glucose [DMEM-LG]; Sigma) for 15 min at 37°C on a nutating shaker, followed by an overnight digest in collagenase type II (583 U/mL in DMEM-LG with 10% fetal bovine serum [FBS]; Worthington).^{12–14} Digests were then filtered through a $70\text{ }\mu\text{m}$ cell strainer, diluted with DMEM-LG, and centrifuged at $700g$ for 10 min. Cell pellets were resuspended in DMEM-LG supplemented with 10% FBS, 1% pen/strep and seeded onto tissue culture flasks at 6000 cells/cm^2 .¹² Cells were trypsinized from flasks during log-phase growth and seeded into $4\times 4\text{ cm}$ bioreactors at $3.125\times 10^6\text{ cells/cm}^2$ in chondrogenic

media (Dulbecco’s modified Eagle’s medium-high glucose [DMEM-HG], 1% ITS+Premix, 1% minimum essential medium non-essential amino acids [MEM NEAA], 1% pen/strep, 1% pyruvate, 1% Glutamax, 0.5% Fungizone, 100 nM dexamethasone, $130\text{ }\mu\text{M}$ ascorbate-2-phosphate) in a 500 mL Nalgene container. Cells were allowed to settle and form a sheet over 2 days before being placed on a rotary shaker with partial media replacement ($\sim 10\%$) every 2 to 3 days. After 9 weeks of culture, twenty-seven 5 mm diameter disks were cut from the TEC sheets using a skin biopsy punch and frozen -20°C until use. A total of 33 disks were used in this study.

For testing, all samples were thawed in room temperature phosphate-buffered saline (PBS) for half an hour before the test. The samples were kept moist with PBS during preparation. The test samples were mounted to magnetic stainless steel sample holders using cyanoacrylate cement (Loctite 4011; McMaster-Carr, Cleveland, OH).

US evaluation

For all US evaluations, a pulser-receiver operating in transmit/receive mode provided excitation and received reflections from the sample (DPR500, JSR Ultrasonics; Imaging, Pittsford, NY). A 30 MHz focused beam transducer (model V375-SU, 19 mm focal length, focused beam diameter $\approx 150\text{ }\mu\text{m}$; Fig. 2B; Olympus, Waltham, MA) was used.

For samples ($n=27$) sheared using the first generation device (system 1; see section “Sliding shear”) US evaluation

FIG. 2. (A) Device used to characterize damage in cartilage samples using US. A sample is attached to a magnetic stainless steel holder and is immersed in PBS, which couples the focused US beam and sample. Reflections from the front surface of the sample and from the interface between the sample and the sample-holder are recorded and analyzed to identify signs of damage. The X-Y stage is used to scan the transducer across the sample. (B) A schematic of the testing device in (A). PBS, phosphate-buffered saline. Color images available online at www.liebertpub.com/tec

was performed using a separate device as described previously.⁹ For samples ($n=11$) evaluated using the second generation device (system 2; also see section “Sliding shear”) an US transducer was incorporated into the sliding shear device.

For all samples, US reflections were acquired before, during, and after the sliding shear from both the sample and the metal sample-holder. If internal discontinuities in the tissue (e.g., cracks) developed as a result of the sliding shear, these were captured as new echoes. It should be noted that reflections off the sample-holder were also captured through the cartilage, confirming that the entire thickness of the sample was penetrated by the US. (We have previously assessed depth of penetration and were able to resolve features through over 6 mm of cartilage up to 50 MHz).

Signals were sampled at 5 GS/s using a digital oscilloscope (Keysight MSOX3054T); these data were stored as comma-separated values (csv) files for analysis, and oscilloscope trace images were stored as portable network graphics (png) files.

Surface roughness

For a subset of samples (11), the transducer was scanned across a sample's surface in 0.5 or 1 mm increments along a single line before and after sliding shear. Undulations in the surface were computed using time of flight (TOF) from the transducer to the cartilage-PBS interface, using the speed of sound (SOS) in PBS as 1495 m/s.¹⁵ We computed an ultrasound roughness index (URI) as described by Laasanen *et al.*¹⁶ and Saarakkala *et al.*¹⁵ using:

$$URI = \sqrt{\frac{1}{n} \sum_{i=1}^n (d_i - \bar{d})^2}$$

where n is the number of measurements across the surface, d_i is the distance between the transducer and the cartilage-PBS interface at each point, and $\bar{d}$ is the average distance. Note that converting TOF to distance depends on SOS in PBS only, which is known, SOS in cartilage is not needed.

Sample thickness

TOF between echoes from the cartilage surface and the cartilage sample holder interface was measured by identifying the time of occurrence of the first peak of each reflection. These data were used to determine if the thickness of a sample had changed after sliding shear. Other approaches for measuring TOF were evaluated using simulated data, and US signals from tissue phantoms, and TEC. For each of these, we compared three approaches: “manually” picking the relevant peaks on the US recording trace, cross-correlation, and Hilbert envelope. For the latter two approaches we used custom MATLAB routines. But, given the small number of data sets in this study and the large time shifts, we used the “manual” approach in this study (see also Supplementary Data; Supplementary Data are available online at www.liebertpub.com/tec).

Surface imaging

Before and after sliding shear, an image of the exposed surface of each sample was obtained using a stereomicro-

scope (EZ4W; Leica Microsystems, Buffalo Grove, IL) and stored as a tagged image format file (TIFF).

Sliding shear

Samples were evaluated in one of two different sliding shear devices. Both operate identically with respect to the sliding shear.

A set of pilot experiments used the first device (system 1) which has been described previously (Fig. 3).^{5,6} Up to four samples were tested at one time. Shear was applied to the samples by an oscillating glass microscope slide counterface in a PBS bath. A new slide was used for each test. Weights ranging from 200 to 1500 g were used to provide the normal force that pressed samples against the counterface. These forces correspond to normal stresses of 0.14–0.79 MPa (Table 1). In all tests with system 1, the length of the oscillation was 0.020 m in each direction, the cycle time was 24.66 s, which corresponds to an average sliding speed of 1.62×10^{-3} m/s.

Samples were tested for 0, 10, 100, or 1000 cycles (Fig. 1, left). However, if tissue debris was observed in the PBS, the failing sample was removed from the test, and the number of cycles was recorded. In the absence of tissue debris observed in the PBS, tests continued to the prescribed number of cycles (Table 1). After the sliding shear tests, the samples were allowed to recover for at least 20 min. They were then photographed using the stereomicroscope; a second set of US data was then recorded.

From these pilot experiments, we noted that surface roughness could be measured. We therefore designed and built a second device (system 2) (Figure 4). This new system uses a single-axis linear slide (XN10-0040-E25-71; Velmex, Bloomfield, NY) driven by a stepper motor (PK245M-01AA; Velmex) to provide oscillating motion. A programmable controller allows us to set the length of travel and speed. The sample was positioned above a dish containing PBS. Function is identical to system 1, except that, in this case, the US transducer was attached directly to the fluid-filled dish. Before each run, the apparatus was calibrated to establish the zero position of the sample holder.

Dead weight (1.0 or 2.0 kg) was used to provide the normal load (Fig. 4). In these tests, the length of the oscillation was 0.025 m in each direction, at a speed of 3.3×10^{-3} m/s for the bovine cartilage and 1.62×10^{-3} m/s for the TEC.

For this set of TE samples (Fig. 1, right), on average, failure occurred after 250 cycles with a 1-kg load as evidenced by debris in the PBS bath. Subsequent samples were thus tested to 75% of this, or 190 cycles. For normal bovine cartilage, samples were run for 1100 cycles under a 2-kg load, with little to no macroscopic evidence of failure at this point. In some samples, there was slight filmy debris in the PBS.

At intervals (Fig. 1, right, loop), and after completion of the sliding shear tests, the samples were allowed to recover for at least 20 min, during which time US traces were obtained from near the center of the sample to document relaxation (Fig. 5). The samples were then scanned at 500 μ m resolution to obtain a surface profile and to document surface roughness (Fig. 6, blue dots).

Samples were processed for Toluidine blue and H&E histology staining. Sections were evaluated and photographed

FIG. 3. Samples were loaded in compression and sliding shear to induce damage. Compression was applied by deadweights placed on a platform above a sample, and the oscillating movement of the table to which the lubricant wells are attached applied shear. Glass microscope slides were used for the sliding counterface. This is a close-up of one loading station, the complete device consists of four of these. Color images available online at www.liebertpub.com/tec

using a microscope. Examples of histology of undamaged tissue are shown in Figure 7.

Evaluation

Visual, photographic, histologic, and ultrasonic data from before and after the sliding shear tests were compared with each other. The US and histology qualitative gradings were done separately and blindly by two investigators (J.M.M. and J.F.W.). Tissue damage was scored on the histology sections using a simple three-level scoring scheme as follows: level 0—no damage, level 1—partial damage, and level 2—complete damage. US traces were evaluated for changes in front reflection amplitude, timing of the front reflection echo, and the appearance of new internal signals. As noted above, for a subset of the samples, URI were computed as well. Finally, in Table 1, a judgment was made whether the changes in US signals were predictive of the damage or lack thereof observed by histology.

Results

Table 1 summarizes all the test parameters, as well as visual, US, and histological results as well as their correlation. An often-observed change between pre- and post-sliding US signals was a decrease or nearly a complete loss of the front surface reflection. Some samples with surface erosion or roughened surfaces following sliding had surface reflections that were reduced, but easily identifiable on an oscilloscope trace (Fig. 8A). Histology (Fig. 8B) confirms that the sample was damaged. In other samples,

surface reflections were so small that additional analysis was needed to identify them (Fig. 9D). In the latter cases, front-surface reflections were found by scanning the transducer relative to the sample to identify the reflection front. In six samples, after sliding shear, there were macroscopically visible new cracks or holes through the cartilage that extended down to the holder (e.g., Fig. 6B, red arrows). Because these were neither within the spot of the US beam (using a focused transducer with a 150 μm spot size; Fig. 6, green dots) nor in the sectioning plane in the histology, they were missed by both (Fig. 9B, C). Widespread damage was also identified using TOF as an indicator of thickness, at 0.5 or 1 mm increments across a sample (Fig. 9E).

As measured by US, 17 samples were thinner after sliding shear, consistent with erosion. This included six of the seven samples tested for 1000 cycles of sliding. Seven samples were thicker overall after shear, consistent with fibrillation of the surface and/or extending to internal delamination opening flaws in the interior of the tissue. In one sample, there was no change in thickness, and in two samples, changes in thickness could not be evaluated, since they sheared off their holders.

It is important to note that in the large majority of samples, where severe damage was observed after the sliding shear test, no visual cues of failure were observed during the tests. In only 8 out of the 38 samples tested, there was tissue debris seen in the well, and the sliding shear was terminated early as a result. This was a small subset of the samples that were severely damaged upon postshear inspection.

TABLE 1. SUMMARY OF LOADING CONDITIONS AND NUMBER OF CYCLES INTENDED/SURVIVED

<i>Sample</i>	<i>Normal force (g)</i>	<i>Normal stress^c (MPa)</i>	<i>Cycles group</i>	<i>Cycles survived^a</i>	<i>US notes</i>	<i>Histology notes</i>	<i>Histo score</i>	<i>Correlates qualitatively</i>
21	600	0.34	10	10	Swelling, increase in front surface signal	Vertical crack, very coarse scalloping, surface otherwise mostly intact	1	Ø
22	800	0.44	10	10	Swelling, attenuation of front signal	Rough, interior intact	1	✓
23	400	0.24	10	10	Swelling, attenuation of front signal	Intact, fibrillated	1	✓
24	200	0.14	10	10	Slight thinning	Specimen lost	N/A	N/A
33	700	0.39	10	10	Compacted, increased front surface otherwise unremarkable	Largely intact	0	✓
34	800	0.44	10	10	Front reflection gone, marked thinning	Destroyed. Many chunks. Hypertrophic tissue	2	✓
35	900	0.49	10	10	Thickness unchanged, slight attenuation of front surface. Internal signal amplitudes increased, loss of signals near holder.	Internal defects, back surface torn up	1	✓
36	600	0.34	10	10	Thinning, slight attenuation of front signal	Coarse scalloping	1	✓
29	1500	0.79	100	2.2	Original front reflection gone, thinning, new internal reflections	Surface shredded, Internal defects	2	✓
30	1000	0.54	100	4.5	No after US, sample fell off holder	Detached sample, one surface intact, one surface very rough	N/A	N/A
32	1200	0.64	100	26	Thickening, surface attenuation	Debris, rough, internal tears	2	✓
31	800	0.44	100	56	Slight thinning, slightly increased front surface reflection	Largely intact	0	Ø
26	400	0.24	100	<100 ^b	Loss of front reflection, scan shows some deep new reflections	One surface intact, one surface shredded with missing chunks, loose debris	2	✓
25	200	0.14	100	100	Large left-shift of signal, new possibly internal signals	Some vertical tears, largely intact. Specimen likely detached from holder	2	✓
27	800	0.44	100	100	Loss of front reflection, marked thinning	Fragments, internal tears, surface OK, but in pieces	2	✓
28	600	0.34	100	100	Loss of front reflection, marked thinning	Both sides very rough, internal tears	2	✓
43	500	0.29	1000	9	Nearly complete attenuation	Vertical tears surface intact	2	Ø
37	200	0.14	1000	1000	Signal essentially gone	Debris only	2	✓
38	200	0.14	1000	1000	Front signal attenuated by half, thinning, internal reflections	Internal tears and defects	2	✓
39	400	0.24	1000	1000	Signal essentially gone	Debris, internal tears, surface destroyed	2	✓
40	400	0.24	1000	1000	Loss of amplitude, thinning	Debris	2	✓
41	700	0.39	1000	1000	Compacted	Internal tears, surface mostly intact	2	Ø
42	600	0.34	1000	1000	Compacted, front surface attenuation	Surface scalloped, one vertical tear off to side	1	✓

(continued)

TABLE 1. (CONTINUED)

Sample	Normal force (g)	Normal stress ^c (MPa)	Cycles group	Cycles survived ^a	US notes	Histology notes	Histo score	Correlates qualitatively
51	0	0.00	0	N/A		Unloaded control	0	N/A
52	0	0.00	0	N/A		Unloaded control	0	N/A
53	0	0.00	0	N/A		Unloaded control	0	N/A
54	0	0.00	0	N/A		Unloaded control	0	N/A
1102	1000	0.75	324	270	Front signal attenuated by half, thickening	Coarse and fine scalloping, vertical tear	1	✓
1031	1000	0.75	224	224	Minimal loss of amplitude, minimal compaction	Intact	0	✓
1108	1000	0.75	190	124	Signal essentially gone	Destroyed. Chunks	2	✓
1109	1000	0.75	190	190	Minimal loss of amplitude	Intact	0	✓
1110	1000	0.75	190	190	Minimal loss of amplitude, minimal compaction	Intact	0	✓
B1116	2000	1.73	1100	1100	Minimal compaction	Intact	0	✓
B1117	2000	1.73	1100	1100	New front surface reflection doublet	Horizontal sub-surface defects	1	✓
B1120	2000	1.73	1100	1100	Front surface minimal loss of amplitude	Intact	0	✓
B1122	2000	1.73	1100	1100	Minimal swelling	Intact	0	✓
1116	0	0.00	0	N/A		Intact, unloaded control	0	N/A
BN1117	0	0.00	0	N/A		Intact, unloaded control	0	N/A

“B” prefix in column 1 signifies normal bovine cartilage; no prefix signifies tissue-engineered cartilage. “BN” is unloaded normal bovine cartilage. ✓ in rightmost column means that US qualitatively predicted histology findings. Ø in rightmost column means that US did not predict histology findings.

^aIf *cycles survived* is less than *cycles intended*, based on seeing loose tissue in a well during sliding, the test for that sample was stopped.

^bDamage noted at 100 cycles, but actual number unknown.

^cStress is based on the applied normal force and the weight of the part on which the normal weight is placed (81 g for system 1—samples 21 through 43, and 496 g for samples 1031 through B1122). The qualitative US and histology gradings were done separately and blindly. N/A, not applicable; US, ultrasound.

FIG. 4. Combined sliding shear and US apparatus. A single-axis linear slide driven by a stepper motor produces oscillating motion of a sliding stage carrying a PBS-filled well with a glass bottom. The sample is fixed in space above the stage; deadweight is used to provide normal force. A single element focused US transducer is attached to the linear slide below the fluid-filled dish and can be moved into position under the sample using the slide. Fine positioning of the sample relative to the transducer is accomplished using the z-axis adjustment micrometer. Color images available online at www.liebertpub.com/tec

FIG. 5. Relaxation of the sample centerpoint after sliding shear, expressed as TOF to front of cartilage (normal bovine cartilage). The parallel but shifted curves suggest a small, but permanent loss of thickness between 100 and 1100 cycles. Before=reference line of sample thickness before loading. TOF, time of flight. Color images available online at www.liebertpub.com/tec

Detecting TOF to front and rear surfaces of the sample

Based on our assessment of methods for determining TOF, we determined that there was little difference between picking the first peak manually and using cross correlation or a Hilbert envelope. For this investigation, the time shifts were so large that little was gained by using automated methods. Therefore, we chose to use “picking the first peak” because for the amount of data involved, this was the simplest approach. Supporting data are shown in Supplementary Data. Going forward, for larger data sets, using cross-correlation will likely be advantageous. It should be noted that cross-correlation outperformed the Hilbert envelope approach for the data evaluated (see Supplementary Data). Table 2 summarizes the sample thickness results before and after sliding shear.

Ultrasound roughness index

Other groups, for example, Laasanen *et al.*¹⁶ and Saarakkala *et al.*¹⁵ have used US surface roughness on damaged

cartilage. Although in a pilot experiment using System 1, this approach looked promising (Table 3, Fig. 10), further investigation of this approach only predicted the histology results if the sample was completely destroyed.

Discussion

Articular cartilage functions as a load-bearing surface in freely movable joints such as the knee and hip. As such, it is loaded by compression and shear forces. Maximum normal force in a joint during activities of daily living is about five times body weight, resulting in normal contact stress of about 10 MPa.^{17–23} In healthy joints, shear forces are one to three orders of magnitude lower than compression forces due to the low coefficient of friction between articulating cartilage surfaces.^{24–27} Articular cartilage is capable of performing its function as a bearing surface for decades, however, due to a lack of intrinsic repair mechanisms, it is susceptible to irreversible damage and degradation.

TEC is emerging a biological means for repairing the degradation of articular surfaces. Unfortunately, experience to date has shown that TEC is not up the task.^{1–6} In particular, previous work in our laboratories has shown that TE constructs have mechanical properties that are much lower (aggregate modulus was 60–70% lower than native cartilage in previous studies) than normal cartilage and that they can be severely damaged under compression and sliding shear loads.^{5,6,11,28,29} Thus, realistically evaluating the performance of engineered cartilage *in vitro* requires subjecting the tissue to combined normal and shear forces.

In the large majority of samples in this and previous studies, although severe damage was observed after the sliding shear test, there were no visual cues of failure during the tests.^{6,11} Conventionally, damage was assessed by histology after a prescribed number of cycles of shear have been applied.^{6,11} The assay would be much improved if damage could be assessed in real or near-real time.

The potential value of using a real-time method to identify damage following sliding shear is illustrated by, for example, Whitney *et al.*,^{14,30,31} who showed that damage in cartilage generated in the presence of Thyroxine is reduced compared with cartilage grown without Thyroxine. Thus,

FIG. 6. (A) Stereomicroscope image of a sample before sliding shear. The surface is relatively smooth and continuous. The colored dots show the size and position of the focused US beam. (B) Stereomicroscope image of a sample after sliding shear. After shear, cracks have opened, which in some cases, extend through the full thickness of the sample (red arrows). These cracks would not necessarily be identified when the sample is interrogated using a single fixed-position element US transducer (green dots) or by histology. When a line-scan across the sample was taken (blue dots), more area is covered, but some damage can be missed. Sample 31 in Table 1. Color images available online at www.liebertpub.com/tec

FIG. 7. Histology of (A) normal bovine cartilage test sample and (B) tissue-engineered cartilage samples. Toluidine blue staining, scale bar is 1 mm. Color images available online at www.liebertpub.com/tec

comparing a sample generated using a new TE protocol with one generated using an existing protocol, both are found to have failed at the end of 1000 cycles, knowing that one failure occurred at 100 cycles and the other at 900 cycles would be an important information in evaluating the new protocol. While this could be achieved using a large number of samples, each analyzed by histology after many different number of cycles, this is impractical and costly.

Although US has been used to characterize TE, native, osteoarthritic, and enzyme-treated cartilage via acoustic properties,^{1,15,32–41} to the best of our knowledge, this is the first study to use US to characterize damage incurred as the result of combined compression and sliding shear. We investigated the feasibility of using US as a nondestructive means for detecting damage in TEC, based on its established use for detecting flaws in engineering materials, its ability to

FIG. 8. (A) US signals before and after sliding shear. Note the attenuation of the amplitude of the reflection from the sample's surface (double-headed blue arrow). There is also a relative shift of the "After" signal to the left of the "Before" signal, suggesting that the sample was thicker after damage (green arrow). A new area of attenuated signal appears in the "After" signal (blue arrow), close to the return from the metal holder (magenta arrow). (B) Histology clearly showing that the sample was damaged. Where the sample has cracked and separated into two legs, it is thicker than the bulk material, which is consistent with the thickening shown by US. Sample 35 in Table 1. Scale bar is 500 μm . Color images available online at www.liebertpub.com/tec

FIG. 9. Stereomicroscope images of the surface before (A) and after (B) sliding. Damage is clearly evident in (B). (C) Histology of the damaged sample. (D) Single point (150 μm diameter spot) US traces before and after sliding shear. The salient feature is the loss of an easily identifiable front face reflection in the “After” (red) trace due to destruction of the surface and scattering of the US wave. (E) When multiple thickness measurements were taken at 1 mm increments across the sample’s surface, the roughness of the surface (red line) can be clearly determined from US TOF. The black line shows the original thickness of the sample. Histology scale bar is 500 μm . Sample 29 in Table 1. Color images available online at www.liebertpub.com/tec

interrogate the entire thickness of a sample, and its relatively low cost. In a first step (system 1), we subjected TEC samples to extensive sliding shear and evaluated the sample immediately after loading using a separate US system. Based on the results of this portion of the investigation, implementing near real-time assessment of damage using US is feasible and preferable to debris detection, and much faster than histology as means of assessing damage. For example, in this part of the investigation, tissue debris was only seen in 6 out of the 23 samples that were tested, whereas 19 out of 23 showed damage by histology. US correctly predicted damage state in 16 out of 23 samples with no false positives. As discussed below in *Limitations*, a single, fixed, focused transducer as used here would be expected to miss some damage, as it does not occur at a single point, but rather it is distributed over the sample, with some areas being more susceptible than others. In addition, with this setup, because of repeatability considerations, it was not practical to perform more than one iteration of sliding shear followed by US measurement.

Based on the limitations of system 1, we then implemented system 2, which integrates the sliding shear and US evaluation. Using this system, we were able to intersperse sliding shear and US repeatedly into one test, and to control the position of the transducer sufficiently to perform a reproducible linear scan of the cartilage sample, without issues of alignment or indexing. Using system 2, US correctly predicted the histological damage state in 9 out of 9 samples. (Two additional samples were undamaged controls).

Using US, several types of signal pattern changes were observed. A common finding was a reduction in the amplitude of the reflection from the surface of a sample following sliding shear. We attributed this to increased microscale surface roughness that would cause scattering of the reflected US wave and, therefore, less of the incident energy was reflected back to the US transducer. This observation is supported by independent measurements of surface characteristics in engineered cartilage. Gelse *et al.*¹ showed that the US Integrated Reflection Coefficient in repaired cartilage that filled surgically created defects in the pig knee decreased relative to native tissue. Using white light interferometry and scanning electron microscopy, Accardi *et al.*² showed increased surface disruption in engineered cartilage following sliding shear. Using cartilage grown from chondrocytes in alginate, Gleghorn *et al.*³ observed minimal surface alterations after sliding shear under low stress, ≤ 10 kPa, but these were not quantified. In a number of samples we observed thickness increases. In several cases, this was attributable to gross internal cracking/delamination of the tissue rather than swelling of the extracellular matrix (ECM) (Fig. 8). However, tissue swelling due to finer disruptions of the ECM architecture has also been described by others and may be contributing here. In many cases, there was minor compaction of the tissue as measured by US. In isolation, this did not correlate with any histological findings and would likely have been reversible if longer relaxation time had been allowed (Fig. 5). At any rate, tissue shrinkage due to the

TABLE 2. SAMPLE THICKNESSES MEASURED BY ULTRASOUND BEFORE AND AFTER SLIDING SHEAR

Sample	Thickness before (m)	Thickness after (m)	Difference (m) ^a
21	1.04E-03	1.19E-03	-1.52E-04
22	5.28E-04	7.39E-04	-2.11E-04
23	3.25E-04	4.05E-04	7.992E-05
24	1.67E-04	1.43E-04	2.35E-05
25	3.53E-04	1.08E-03	-7.28E-04
26	4.84E-04	0.00	Unknown
27	5.64E-04	2.76E-04	2.87E-04
28	4.03E-04	0.00	Unknown
29	9.94E-04	0.00	Unknown
30	3.02E-04	“After” data missing	Unknown
31	2.42E-04	2.19E-04	2.27E-05
32	7.53E-04	8.79E-04	-1.26E-04
33	2.71E-04	2.41E-04	3.00E-05
34	3.92E-04	1.18E-04	2.74E-04
35	4.03E-04	4.16E-04	-1.36E-05
36	1.52E-04	1.49E-04	3.34E-06
37	1.57E-04	1.63E-04	-6.66E-06
38	5.40E-04	5.21E-04	1.84E-05
39	6.00E-04	0.00	Unknown
40	3.70E-04	3.43E-04	2.73E-05
41	5.06E-04	3.98E-04	1.0823E-04
42	4.06E-04	2.66E-04	1.40E-04
43	3.03E-04	2.04E-04	9.94E-05
1031	4.09E-04	3.41E-04	6.80E-05
1102	3.33E-04	3.18E-04	1.51E-05
1108	2.19E-04	No signal	Unknown
1109	4.31E-04	4.09E-04	2.27E-05
1110	3.11E-04	3.07E-04	4.73E-06
B1116	10.33E-04	11.13E-04	-7.95E-05
B1117	12.87E-04	10.33E-04	2.54E-04
B1120	No thickness data	No thickness data	Unknown
B1122	7.52E-04	1.10E-03	-3.45E-04

^aNegative thickness difference indicates thicker sample.

fixation and dehydration process would make a histological thickness evaluation unreliable. Finally, in some samples, new internal reflections, and/or internal regions of signal attenuation appeared after sliding shear. These correlated with internal cracks by histology (Fig. 8).

Although the stress range in these measurements was considerably lower than the expected values for normal activities of daily living, samples were still damaged. We did not observe any correlation between normal force and damage suggesting that there is considerable variation in engineered cartilage. This observation is consistent with the variability that has been observed in engineered cartilage in other studies.^{42,43}

Methods other than US were considered and rejected for this application. Thickness and surface roughness might be measured using dial indicators or calipers, but these have much lower resolution, and can potentially damage the tissue. Alternative noncontact method approaches, including magnetic resonance imaging, confocal or two-photon microscopy, surface profilometry, and scanning electron microscopy, were rejected based on their cost and difficulty implementing them in a sliding-shear device, especially for near real-time measurements of damage.^{2,44–48} Approaches such as reflected polarized light microscopy provide information on surface degradation, but do not address internal delamination.⁴⁹ Measuring collagen or proteoglycan concentration in the lubricant is a potentially valuable indicator of damage, but given the gross damage seen in our tests, it is not needed at this time.⁴⁶ However, as new methods for producing TEC with improved mechanical integrity as developed, biochemical indicators of wear may become more important.

Limitations

TOF calculations for thickness were made under the assumption that SOS does not change over the course of the sliding shear test. While this assumption is often invoked, it may introduce error in length measurements.^{15,16,50,51}

Since, as implemented in our device, the US scan occurs along a single line, with measurement separated by ~0.5 mm, we may have missed damage that was present, but at a scale below the resolution of our system.² This also applies to the URI computations made with data from a single-line scan. Again, given the gross damage sustained by these samples, finer resolution may not be needed at this time. Identification of focal defects would be greatly improved by using an X-Y scanning system or an US transducer array that is at least as long as the diameter of a sample, and that is incrementally scanned across a sample.

TABLE 3. ULTRASOUND ROUGHNESS INDICES

Sample	URI Pre	URI 4	URI 24	URI 100	URI 124	URI 190	URI 224	URI 270 ^a	URI 1100
29 ^b		3.324E-06							
1031	3.233E-08	8.155E-08	6.850E-08		7.425E-08		1.301E-07		
1102	5.625E-08	4.458E-08			3.910E-08		3.546E-08	1.359E-07	
1108	6.201E-08	6.496E-08	6.054E-08		1.075E-07				
1109	2.578E-08	1.786E-08	1.623E-08		8.829E-09	1.625E-08			
1110	8.348E-08				2.699E-08	1.898E-08			
B1116	2.651E-07			1.834E-07					1.645E-07
B1117	5.608E-07			9.118E-07					4.955E-07
B1120	3.936E-07			3.692E-07					1.931E-07
B1122	3.289E-08			6.565E-08					5.013E-08

These were computed using the first peak approach as described in Materials and Methods. Loading parameters are shown in Table 1.

^aSample failed at 270 cycles, 324 cycles were planned.

^bPilot sample scanned at 1 mm intervals using system 1. Sample failed catastrophically at 2.2 cycles.

URI, ultrasound roughness index.

FIG. 10. Surface profiles of two TE and one native bovine cartilage samples taken before and after the indicated number of cycles showing different types of surface changes. **(A)** Native bovine cartilage before and after 100 and 1100 cycles showing no significant changes (sample 1117). **(B)** TEC showing slight compaction at the center of the sample, slight swelling elsewhere (sample 1110). **(C)** Sample showing marked increase in thickness. Histology shows tissue to be broken up with multiple cracks (sample 1108). Color images available online at www.liebertpub.com/tec

Summary

In summary, we have shown that US can detect damage to TE articular cartilage that was subjected to compression and sliding shear. Attenuation of the reflection from a sample's surface and changes in thickness were the clearest indicators of damage. An important observation is that gross damage does not occur at a single point, but rather it is distributed over the sample, with some areas being more susceptible than others. A single fixed-focused transducer as used in the early part of this feasibility study would not, in general, be able to detect all sites of distributed damage. Scanning the transducer across the surface in a single direction at 500 μm steps improves the quality of the data acquired, but still can miss localized damage. We should note that the same limitation applies to the current gold standard of analysis, histology, where the placement and orientation of the sectioning plane defines what portion of the sample will be analyzed. In contrast, an US transducer array or a mechanical X-Y scanning system could scan the entire surface of a sample and thus could identify damage at any location. Development of such a system carries a large cost in terms of hardware and software development, but given the findings presented here, we feel that this will be a worthwhile addition to the toolset for evaluating engineered cartilage.

Acknowledgments

This work was supported by the National Institute of Biomedical Imaging and Bioengineering under award

number P41 EB021911. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. Dr. Motavalli was supported by funds from the Office of Research and Technology, and the deans of the College of Arts and Sciences, the School of Engineering and the School of Medicine at Case Western Reserve University.

Disclosure Statement

No competing financial interests exist.

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Received: December 13, 2017

Accepted: June 25, 2018

Online Publication Date: August 3, 2018