

# Effects of directed gel degradation and collagenase digestion on the integration of neocartilage produced by chondrocytes encapsulated in hydrogel carriers<sup>‡</sup>

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## Abstract

Chondrocytes were encapsulated in non-degrading and partially degrading poly(ethylene glycol) (PEG) gels in apposition to native cartilage layers in order to examine the effects of gel degradation on the integration of regenerated cartilaginous matrix with native tissue. In addition, the effect of collagenase predigestion of the native cartilage surfaces on this integration was examined in studies with partially degrading co-polymer gels. Integration was quantitatively assessed by mechanical measurements of adhesive strength, and visualized by histological staining and non-destructive ultrasound analysis. Constructs with encapsulated chondrocytes and a non-degrading gel layer had significantly higher adhesive strength than partially degrading gel constructs and non-degrading gel constructs without cells. In addition, better maintenance of proper cell morphology was observed near the gel–cartilage interface in non-degrading gel constructs than in partially degrading gel constructs after 8 weeks of *in vitro* culture. Facile collagen distribution in the degrading gels appeared to have a significant effect on mechanical adhesion measurements only when the native cartilage surface was predigested with collagenase. Ultrasound analysis provided qualitative evidence of cartilaginous matrix evolution and non-destructive imaging of developing constructs and the interface between newly formed matrix and existing cartilage tissue. Copyright © 2008 John Wiley & Sons, Ltd.

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## 1. Introduction

A number of challenges must be overcome before successful treatment of cartilage defects with tissue-engineering strategies becomes common practice. It is clear that long-term integration of regenerated cartilage with native tissue is an important problem with current

therapies, and an area in which tissue-engineering solutions must succeed in order to progress to common clinical application (Buckwalter and Mankin, 1997; Hunziker, 2001). Mature cartilage is an avascular tissue, composed of chondrocytes embedded in an extracellular matrix (ECM) that is mostly water, with type II collagen and proteoglycans making up the majority of the rest of the ECM (Heinegård and Paulsson, 1987). While the lack of vascularity decreases the complexity of engineering new tissue, it increases the complexity of integrating that tissue into a defect site. While many tissues benefit from an escalated blood supply during wound healing, it is unclear whether increased blood supply improves

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the long-term outcome of damaged cartilage (Hunziker, 2001).

A number of previous studies have indicated various steps that can be taken to improve the integration of engineered neotissue and native cartilage. Many of these studies contribute not only to improvements in integration for a particular scaffold or cell carrier, but also to a general understanding of the details that are most important to the process. One approach has been to briefly expose cartilage surfaces to an enzyme that can partially degrade the native matrix. These include cartilage-specific enzyme treatments with chondroitinase and collagenase, as well as non-specific enzymes that cleave many proteins at the tissue surface (Hunziker and Kapfinger, 1998; Obradovic *et al.*, 2001; Giurea *et al.*, 2002; Bravenboer *et al.*, 2004). Both proteoglycans and collagen have been implicated in the integrative process (Dimicco and Sah, 2001; Dimicco *et al.*, 2002; Giurea *et al.*, 2002; McGowan and Sah, 2005). Proteoglycans in the native tissue have been shown to impede the adhesion and migration/diffusion of cells and matrix proteins (McCarthy *et al.*, 1996; Peretti *et al.*, 1998). These negatively charged molecules may intuitively be reduced in concentration near the surface, either by treatment with an enzyme that directly cleaves them into small pieces, or potentially by a collagenase enzyme that opens up the entire ECM structure of cartilage tissue near the surface. Clearly, collagen produced in engineered tissue must be able to partially diffuse into the native ECM in order for the interface to structurally remodel and be replaced by homogeneous tissue. Enzyme pretreatment may be necessary to open up the ECM structure of cartilage and allow interconnection of new and existing tissue on a macromolecular basis.

Another approach has been to improve the adhesive bond at the interface of two tissue samples (as present in transplantation surgeries) or a tissue sample with a biomaterial scaffold or cell carrier. Some researchers have focused on improving this adhesion with a biological adhesive, such as tissue transglutaminase (Jurgensen *et al.*, 1997; Ahsan and Sah, 1999) or fibrin glue (Peretti *et al.*, 1999). Others have opted for more complex solutions that link native tissue to a biomaterial via covalent bonds with collagen crosslinkers (Dimicco *et al.*, 2002) or tissue-initiated photopolymerizable biomaterials (Wang *et al.*, 2004). The general idea behind these approaches is that improvement of initial adhesion will prevent slipping and keep the important interface in stable apposition, allowing ECM molecules to bridge across and form a homogeneous tissue. Based on the results of these studies, initial adhesion appears to be an important aspect of successful integration.

One of the advantages of a hydrogel cell carrier is that, to a certain extent, the crosslinked polymer network mimics the type II collagen network of native cartilage tissue. Previous studies by our group have investigated the way that the initial density and degradation characteristics of crosslinks can affect cartilaginous matrix evolution in a developing cell–gel construct (Bryant and Anseth, 2002, 2003). Briefly, collagen tends to have especially

poor distribution until crosslinks degrade to the point that collagen can readily diffuse and begin to replace eroded gel crosslinks with a new fibrillar network. Controlling the crosslinking of the synthetic network and its replacement by neotissue will be especially critical at any interfaces between a cell–gel construct and native tissue. The studies described in this contribution examine this problem in the interfacial region between cartilage disks and a poly(ethylene glycol) (PEG)-based hydrogel with encapsulated chondrocytes. By combining our knowledge and control of gel degradation with enzymatic pretreatments and characterization techniques, we quantitatively and qualitatively compare the integration and adhesion of the native tissues with regenerated matrix in the hydrogel biomaterials. We hypothesize that modifications to gel degradability affect distribution of matrix components in the hydrogel and superficial tissue region, and that the presence of distributed matrix components has a significant effect on adhesion and integration, as described or verified by mechanical testing and histology.

It is also apparent that a non-destructive analysis technique has a high potential for improving the characterization of developing neotissue at interfaces with native tissue. Ultrasound is a non-destructive analytical technique that can be used to acquire real-time images and quantitative measurements on the microscale level. Ultrasonic propagation and scattering properties depend on the structure and composition of a particular tissue (Bamber, 1986; Shung, 1993). In particular, a strong ultrasonic echo is produced at interfaces of materials with dissimilar elastic properties. Using this echo, interfaces can be visualized in cross-sectional images and a reflection coefficient can be calculated. The reflection coefficient has been used to assess superficial degradation in a number of previous studies aimed at characterizing the ultrasonic properties of normal and diseased cartilage (Toyraas *et al.*, 1999; Laasanen *et al.*, 2003; Saarakkala *et al.*, 2003; Nieminen *et al.*, 2004; Saarakkala *et al.*, 2004). We hypothesize that ultrasound data collected with a scanning acoustic microscope will provide qualitative information through cross-sectional images, and also that a reflection coefficient can be calculated to provide a quantitative measure of the integration of newly formed matrix with existing tissue.

## 2. Materials and methods

The experiments described in this section are based on synthesis and analysis of two- and three-layer constructs with hydrogel and cartilage tissue layers. To clarify the distinction between these constructs, three-layer constructs were necessary for mechanical testing, while two-layer constructs were necessary for ultrasound analysis and convenient for histological analysis. Mechanical testing required a top and bottom cartilage layer that could be affixed to the mechanical tester surfaces. For ultrasound analysis in its presented

form, a top cartilage layer caused significant decay of the ultrasound signal, necessitating analysis of two-layer constructs with the hydrogel layer on top. Finally, two-layer constructs were more convenient for histological analysis because dehydration and rehydration during processing only caused stress from uneven water content on one interface instead of two. This resulted in histological images from two-layer constructs that are most likely to be representative of the actual tissue–hydrogel interfaces.

## 2.1. Dissection of native tissue and isolation of chondrocytes

Chondrocytes were isolated from the femoral-patellar groove and femoral condyles of knee joints harvested from a young calf (Research 87\*<sup>1</sup>, Marlboro, MA, USA). Cartilage chunks were excised under aseptic conditions and digested on an orbital shaker at 37 °C by a solution of 0.2% collagenase type II (Worthington\*) and 5% fetal bovine serum (Invitrogen\*) in Dulbecco's modified Eagle's medium\* (DMEM, Invitrogen\*) without additives. After a digestion period of 15–17 h, the solution was centrifuged at 1200 r.p.m. for 10 min; the supernatant was aspirated off; the cells were resuspended in warm phosphate-buffered saline (PBS; Invitrogen\*; pH 7.4) supplemented with 1% penicillin/streptomycin (P/S; Invitrogen\*) and 0.02% ethylenediaminetetraacetic acid (EDTA; Aldrich\*); and this cell suspension was then filtered through a 100 µm cell strainer. The cells were then centrifuged and resuspended in PBS with 1% P/S twice more. The cells were counted and the viable fraction was determined by use of Trypan blue staining and a haemocytometer.

Cartilage disks were also dissected from the femoral-patellar groove and femoral condyles of a separate young calf knee joint. The joint was sawn into small osteochondral chunks that were then mounted on the movable stage of a Vibratome 1000 Plus\* sectioning system (Vibratome\*, St. Louis, MO, USA). With the use of the vibratome, cartilage slices were cut from the chunks in 800 µm sections. This thickness was determined to be approximately the minimum thickness necessary for easy mounting of three-layer constructs in either mechanical testing configuration, described below. Slices contained cartilage from non-specific depths within the cartilage tissue, with the exception that the slices did not come from the topmost layer (approximately 1 mm) of the harvested cartilage chunks. Disks were removed from the tissue slices with a sterile, circular punch (approximately 4.6 mm in diameter) and stored for up to 1 day in culture medium (described below) in a sterile, humid

environment at 37 °C and 5% CO<sub>2</sub>. Disks prepared for use in three-layer constructs had a 1.5 mm diameter hole punched from the centre of the disk to create an annular geometry, in order to improve manipulation of cartilage layers and to provide gel layers of consistent thicknesses.

## 2.2. Preparation of macromonomer solutions

Linear poly(ethylene glycol) (Fluka\*) with an average molecular weight of approximately 4600 Da was used to synthesize poly(ethylene glycol) dimethacrylate (PEG-DM), as well as a tri-block co-polymer, poly(lactic acid)- $\beta$ -poly(ethylene glycol)- $\beta$ -poly(lactic acid) dimethacrylate (PEG-LA-DM), as described previously (Sawhney *et al.*, 1993). The PEG-DM macromonomer forms crosslinks that are stable on the timescale of years *in vitro*, and are therefore referred to as non-degrading on the timescale of these experiments. The PEG-LA-DM macromonomer forms crosslinks that degrade completely in the timescale of these experiments. Proton NMR analysis of the PEG-DM macromonomers revealed approximately 85% methacrylation of end groups, and >95% methacrylation of end groups and an average of six lactic acid repeat units per side of the PEG core for the PEG-LA-DM macromonomers. To prepare macromonomer solutions, PEG-DM or a combination of PEG-DM and PEG-LA-DM was dissolved in sterile PBS to a final concentration of 10% by weight. For co-monomer mixtures, 25 mol% of the macromonomer was PEG-DM and the remaining 75 mol% was PEG-LA-DM. Gels formed from PEG-DM macromonomer only are referred to as non-degrading gels. Co-polymer gels formed from a combination of PEG-DM and PEG-LA-DM macromonomers are referred to as partially degrading gels. The UV photoinitiator, 2-hydroxy-1-[4-(hydroxyethoxy) phenyl]-2-methyl-1-propanone (D2959; Ciba-Geigy\*), was added to a final concentration of 0.05% by weight. For constructs with cells, chondrocytes were added to sterile macromer/initiator solutions to a final density of  $7.5 \times 10^7$  cells/ml.

## 2.3. Preparation and culture of two- and three-layer constructs

Three types of hydrogel conditions were used for two- and three-layer constructs. These types were non-degrading gels without cells, non-degrading gels with cells, and partially degrading gels with cells. Schematics of the two- and three-layer set-up are shown in Figure 1. All constructs were synthesized in sterile 1 ml syringes with the tips removed. For two-layer constructs, a cartilage disk was placed on the surface of the plunger; 30 µl of the appropriate cell/macromonomer suspension were added to the syringe, completely covering the cartilage surface. The solution was then polymerized under 365 nm UV light for 10 min at an intensity of approximately 10 mW/cm<sup>2</sup>, approximate conditions previously found to

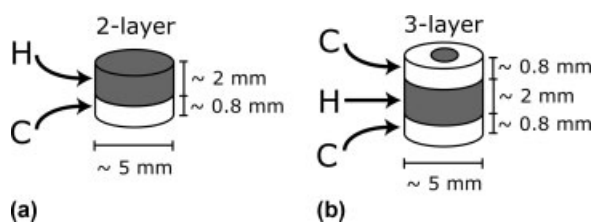
<sup>1</sup> \*Certain commercial entities, equipment, products or materials are identified in this document in order to describe a procedure or concept adequately or to trace the history of the procedures and practices used. Such identification is not intended to imply recommendation, endorsement or implication that the entities, products, materials or equipment are necessarily the best available for the purpose.

be cytocompatible (Bryant *et al.*, 2000). For three-layer constructs, a cartilage annulus was placed on the plunger, 30  $\mu$ l cell/macromonomer suspension were added, and another cartilage annulus was placed on top of the liquid layer. By use of the hole in the middle of the annular shape, the cartilage layers were aligned as parallel as possible, and the syringes were placed on their side under the same UV light, also for 10 min, at an intensity of approximately 10 mW/cm<sup>2</sup>. For constructs with predigestion by collagenase type II, disks were incubated for 15 min on an orbital shaker (40 r.p.m.) at 37 °C in a 0.2% collagenase solution identical to that used for chondrocyte isolation. Only three-layer constructs were synthesized for the collagenase studies, and with only one hydrogel condition: partially degrading gels with cells.

Following polymerization, all constructs were incubated in untreated 24-well plates at 37 °C and 5% CO<sub>2</sub> in a humid environment. During incubation, the plates were placed on an orbital shaker rotating at 40 r.p.m. Constructs were cultured in DMEM\* supplemented with 1% P/S, 0.02 mg/ml gentamycin (Invitrogen\*), 10 mM HEPES (Invitrogen\*), 0.1 mM MEM non-essential amino acids (Invitrogen\*), 0.4 mM L-proline, 0.05 mg/ml L-ascorbic acid, 0.5  $\mu$ g/ml fungizone (Invitrogen\*) and 20% fetal bovine serum (Invitrogen\*). Constructs received 2 ml fresh media every 3–4 days.

## 2.4. Mechanical measurement techniques

Mechanical testing was performed on three-layer constructs after 0, 4 and 8 weeks of *in vitro* culture time. For constructs in collagenase studies, measurements were taken after 4 and 8 weeks of *in vitro* culture time. Measurements were made with a Synergie 100\* mechanical tester (MTS\*) in simple tensile mode. The outer cartilage layers of all constructs were fixed on the top and bottom to separate Delrin® \* (DuPont\*) parallel plates attached to the mechanical tester, and then axially extended at a rate of 1 mm/min. The adhesive strength of the interface was calculated for each sample by dividing the force



**Figure 1.** Description of two-layer constructs used for histological and ultrasound analysis (a) and three-layer constructs for mechanical analysis in all studies and histological analysis in studies of collagenase predigestion (b). Constructs were synthesized with hydrogel (H) layers consisting of non-degrading gels, non-degrading gels with encapsulated chondrocytes, and partially degrading co-polymer gels with encapsulated chondrocytes, polymerized in apposition to excised cartilage tissue (C) layers. In collagenase predigestion studies, one set of cartilage layers were predigested with 0.2 wt% collagenase type II for 15 min at 37 °C

at breaking by the interfacial area, approximated by the cartilage surface area of a complete and intact explanted tissue disk. The surface area resulting from the presence of the opening on cartilage layers was not used as part of the calculations, because the extent to which it was filled with hydrogel was not consistent and the measurements were used only for relative comparisons. For 0 and 4 week time points and for all time points on constructs in collagenase studies, the outer cartilage layers were fixed to the plates with a cyanoacrylate adhesive. The cartilage layers of the 8 week, non-collagenase studies were held in place by four evenly-spaced syringe needles, mounted through 10 mm diameter Delrin rings that were superglued to the top and bottom Delrin plates. The syringe needles were physically pushed through the Delrin rings and into the outer cartilage layers to hold them in place. This change was made in anticipation of the adhesive strength of the cartilage–hydrogel and neotissue interface being greater than the adhesive strength of the cartilage–Delrin interface. The change in measurement configuration affected only the manner in which the outer cartilage layers were fixed to the plates and did not affect the failure of the cartilage–hydrogel interface.

## 2.5. Histological staining

At pre-selected time points of 4 and 8 weeks, constructs were removed from culture, fixed in 10% formalin for 8–24 h, dehydrated, paraffin-embedded and microtomed into 8  $\mu$ m sections. The sections were stained without further treatment with fast green and safranin-O, which stains glycosaminoglycans (GAGs) red-orange, or Masson's trichrome, which stains collagen blue.

## 2.6. Ultrasound analysis

Measurements were performed with an acoustic microscope system in double transmission and pulse-echo modes to measure, respectively, the propagation and backscatter properties of the cartilage specimens. The transducer (Model MSIC-50M S-12\*, Sonix Inc., Springfield, VA, USA) had a nominal centre frequency of 50 MHz, peak frequency of 40 MHz, –6 dB bandwidth of 40 MHz (25–65 MHz), pulse duration of 80 ns, beam diameter of 90  $\mu$ m, and a depth of field of 1.2 mm. The ultrasonic pressure field travelled through buffered saline maintained at 37 °C before interacting with the tissue. Cartilage tissue specimens were held in position by a custom tissue specimen fixture, such that the back wall of the thin specimen was placed at the nominal focal plane of the transducer. The transducer was attached to an automated three-axis motion control system that permitted spatial mapping of the ultrasonic information. The transducer raster scanned across the specimen in step sizes determined to be approximately half the beam diameter. The depth within the two-layer construct at which the ultrasonic field was focused was adjusted by modifying

the distance between the transducer and the front wall of the construct.

We implemented standard data reduction methods in Matlab\* (The MathWorks Inc.\*, Natick, MA, USA) to calculate the amplitude reflection coefficient (Kinsler *et al.*, 2000), which is basically a measure of the fraction of ultrasound that is reflected from an interface with unmatched acoustic impedances. Briefly, the reflection coefficient was calculated by normalizing the power spectrum of the reflected ultrasound from the hydrogel–cartilage interface by the power (or magnitude) spectrum of the reflected ultrasound from the stainless steel plate. The power spectra were estimated by standard Fourier techniques applied to the ultrasonic time-domain echoes.

## 2.7. Statistical analysis

Statistical analysis was performed using single-factor analysis of variance with a confidence interval of 0.05. All values in this text are reported as the average plus or minus one standard deviation (SD).

## 3. Results

### 3.1. Mechanical and histological evaluation

Multilayered constructs were synthesized with hydrogel layers consisting of non-degrading gels, non-degrading gels with encapsulated chondrocytes, and partially degrading co-polymer gels with encapsulated chondrocytes. Three-layer constructs were extended in mechanical tests, and the results of these mechanical tests are shown in Figure 2 for each construct type after 0, 4 and 8 weeks of *in vitro* culture. Neotissue is defined as any new tissue formed from secreted matrix components during the course of the experiments. This may come from cells in the hydrogel or cells in the native tissue, near the interface, as no distinction could be made between these

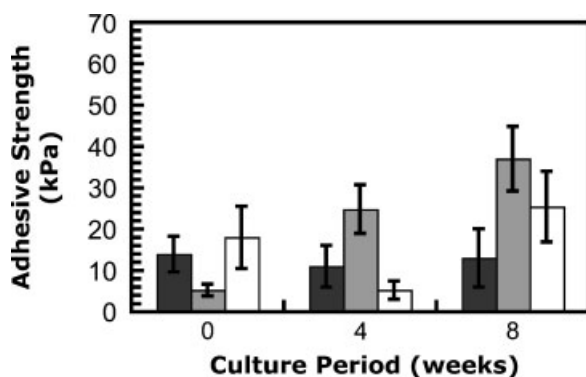


Figure 2. Measured adhesive strengths of neotissue–native tissue interfaces as a function of *in vitro* culture period. Data correspond to non-degrading constructs without encapsulated chondrocytes (dark grey), non-degrading constructs with encapsulated chondrocytes (light grey), and partially degrading constructs with encapsulated chondrocytes (white)

two cell populations in the experiments described here. These measurements of adhesive strength provide relative information on the strength of interfaces as a function of culture time and among different conditions. The first thing to note from these data is the lack of a consistent starting measure, which is most likely due to differences in the methacrylation of the macromonomers that is described in Materials and methods.

Of greater importance is examining the change in the adhesive strengths with *in vitro* culture time for each condition. The non-degrading construct without cells, as expected, did not significantly change over the course of the 8 week experiment, but did show a baseline level of adhesive strength between the polymerized macromonomer solution and native tissue. The measured adhesive strengths of the non-degrading constructs with cells did change. For the periods 0–4 weeks and 4–8 weeks, these constructs showed significant increases in the measured adhesive strengths, from 5 to 25 kPa, and 25 to 37 kPa, respectively ( $p < 0.02$ ). This result indicates that matrix molecules being secreted by encapsulated cells improve the integration of the cell–gel construct with native tissue. Partially degrading gels with encapsulated chondrocytes show more complex behaviour. From 0 to 4 weeks, the adhesive strength between the neotissue and excised cartilage layer decreased from 18 to 5 kPa ( $p = 0.02$ ). The adhesive strength of these constructs increased from 4 to 8 weeks from 5 to 25 kPa ( $p = 0.001$ ) in a magnitude similar to that seen in the non-degrading constructs (with cells) from 0 to 4 weeks. While degradation of the polymer network dominated the effects on measured adhesive strengths from 0 to 4 weeks, neotissue formation by secreted matrix molecules appeared to allow the interfaces to become better bonded from 4 to 8 weeks. Constructs from these experimental groups were histologically stained in order to define which matrix molecules' secretion and distribution at the interface were responsible for changes in the adhesive strengths for each experimental condition.

Figure 3 shows staining of histological sections with the Masson's trichrome method for collagen and with safranin-O/fast green for proteoglycans in non-degrading and partially degrading two-layer constructs after 8 weeks of *in vitro* culture. While the interface appears detached in the non-degrading gels (Figure 3a–b), this is an artifact of the dehydration and rehydration associated with the histological processing. The same artifact was seen in the partially degrading gels, but to a much lesser extent (data not shown). Collagen is well distributed in the evolving tissue layer of the partially degrading constructs (Figure 3c), but localized to the pericellular region in the neotissue layer of the non-degrading constructs (Figure 3a). Proteoglycans appear to be well distributed in evolving tissue layers of both types of constructs with encapsulated chondrocytes. These data for the non-degrading constructs indicate that secretion and accumulation of proteoglycans is a major factor in the development of a bond between the neotissue and excised cartilage layers. However, since the adhesive

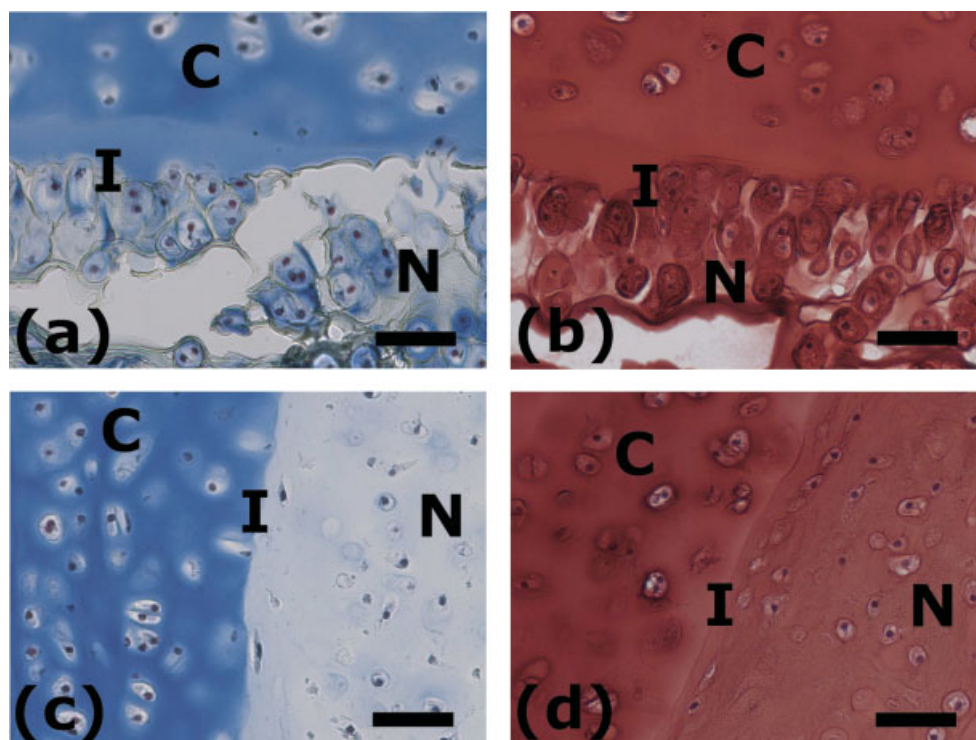


Figure 3. Masson's trichrome (a, b) and saffranin O/fast green (b, d) histological staining of two-layer construct sections after 8 weeks of *in vitro* culture. Sections are from constructs with non-degrading (a, b) and partially degrading (c, d) co-polymer constructs. Labels refer to the excised cartilage layer (C), neotissue layer (N) and the interface (I) between the two. Collagen is stained blue with Masson's trichrome, and glycosaminoglycans are stained orange with saffranin O/fast green. Scale bars = 50  $\mu\text{m}$

forces at the interface in the partially degrading constructs were relatively lower than those for the non-degrading constructs with cells at 4 and 8 weeks, it is impossible to separately discern any effects that the distributed collagen may have had on the integration, at least from a mechanical standpoint.

One should also note the morphology of the excised cartilage tissue directly at the interfaces in Figure 3. In partially degrading constructs, the cartilage surface remains fairly smooth and straight, and is especially easy to observe in the collagen stain (Figure 3c). Near this surface, in the cell-hydrogel layer, the chondrocytes are less rounded than those in the interior of the excised cartilage. The situation is very different in the constructs with non-degrading gel layers, however. The surface of the cartilage layer appears rough, as though it has been partially remodelled by cells at the interface. The encapsulated chondrocytes in the gel layer, near the interface, are large and rounded, very similar to chondrocytes in the interior of the excised cartilage.

In a separate experiment, excised cartilage disks were treated with a 0.2 wt% collagenase type II solution for 15 min prior to synthesis of three-layer, partially degrading constructs. By briefly digesting the surface cartilage matrix, it was expected that the superficial collagen network would be partially degraded and therefore more susceptible to integration with newly produced collagen from the neotissue layer. Ultrasound testing and analysis of the amplitude reflection coefficient

was applied as one method to characterize changes in the initial cartilage surface resulting from this pretreatment. Values for this reflection coefficient were  $0.11 \pm 0.02$  for untreated samples and  $0.04 \pm 0.01$  for samples treated with collagenase. The decreased reflection of ultrasound from the surface of treated cartilage samples most likely indicated a lower density of collagen near the surface of the tissue. This result is corroborated by histological staining of collagen, shown in Figure 4 for both untreated and treated constructs after 8 weeks of *in vitro* culture. Lighter staining appears in a region on the order of 200  $\mu\text{m}$  from the surface of the cartilage layer in the pretreated sample. In addition, chondrocytes near the interface in the neotissue layer appear more rounded and, in some regions, able to modify the cartilage surface.

The mechanical effects of the collagenase predigestion step on the adhesive strength of the interface are shown in Figure 5. Unfortunately, initial measurements were not taken, so it is impossible to describe any initial effects of predigestion on the initial adhesion between gel and tissue layers. As seen in the studies presented above, the adhesive strength is relatively low after 4 weeks of *in vitro* culture, presumably because the gel has significantly degraded. By 8 weeks, the constructs with a pretreated cartilage layer have significantly higher adhesive strength compared to that of untreated controls ( $p < 0.001$ ), indicating that a better bond was formed between the native tissue and diffusing matrix when the density of the collagen network was decreased prior to polymerization to form the two-layer construct.

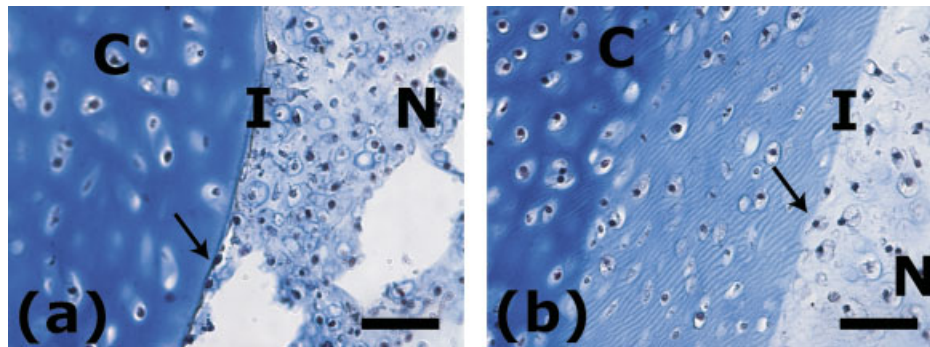


Figure 4. Masson's trichrome staining of three-layer partially degrading constructs after an *in vitro* culture period of 8 weeks, in which the excised cartilage disks were not (a) or were (b) pretreated with collagenase type II. Labels refer to the excised cartilage layer (C), neotissue layer (N), and the interface between the two (I); arrows indicate cells in the hydrogel layer near the interface. Scale bars = 50  $\mu\text{m}$

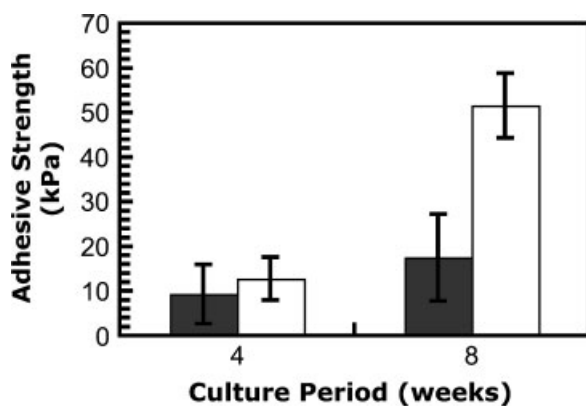


Figure 5. Measured adhesive strengths of neotissue–native tissue interfaces as a function of *in vitro* culture period in three-layer partially degrading constructs without (dark grey) or with (white) pretreatment of the excised cartilage layers with collagenase type II

### 3.2. Ultrasound analysis of the cartilage–neotissue interface

For experiments without collagenase pretreatment, the interface between excised cartilage and evolving cartilaginous matrix was non-destructively assessed both qualitatively and quantitatively with a scanning acoustic microscope. The scanning functionality was applied to probe an area 5.5–6 mm square at a rate that allowed collection of ultrasound data in increments of 100  $\mu\text{m}$ . This range was always adjusted to cover the entire area of the two-layer constructs being tested. Figure 6a shows an A-scan of a signal from one 100  $\times$  100  $\mu\text{m}$  data point for a two-layer partially degrading construct soon after synthesis of the construct (0 weeks). Signals reflected from higher layers of the construct were returned to the transducer with shorter times of flight, resulting in the signal plot shown in Figure 6a. Moving from left to right, the first peak in this plot came from the top surface of the hydrogel layer (G). This peak is very small because the density and speed of sound in the gel are dominated by its water content at early culture times, and impedance in the gel is therefore very similar to that in the buffer solution.

The next peak is from the interface (I) of the hydrogel layer with the excised cartilage layer, and this peak is much larger at this initial time point. The impedances of the cartilage layer and the hydrogel layer are initially very different, but the goal is obviously for the properties of the engineered tissue to eventually match the properties of the native tissue, and integrate seamlessly. The peak in the ultrasound signal from this interface was therefore measured non-destructively with culture time and used to calculate a reflection coefficient. The remaining peaks correspond to the back wall (BW) of the cartilage layer and the stainless steel plate (S). The gap between the last two peaks indicates a small gap of buffer solution between the back wall of the cartilage and the stainless steel plate at this particular data point.

The image shown in Figure 6b is a cross-sectional image of one partially degrading construct at 0 weeks of culture time. The amplitude values of the individual A-scans were mapped to a grey scale to form each column on this B-scan image. Higher magnitudes are represented as light grey to white, so each of the light lines in Figure 6b corresponds to an interfacial signal described earlier. Figure 7 shows the evolution of B-scans with time in two-layer partially degrading constructs and the corresponding accumulation of proteoglycans indicated by safranin-O/fast green histological staining. Note from the B-scans that the signal from the top layer of the gel appears to become larger with time, while the signal from the interface of the hydrogel and excised cartilage appears to become relatively smaller over an additional *in vitro* culture period. The change in signal from the interface may indicate integration of regenerated matrix with the excised cartilage tissue layer, but it is difficult to decipher from the B-scan images alone, as the evolution of distributed cartilage matrix seen in Figure 7c–d may complicate interpretations of the interfacial signal. This signal was therefore analysed mathematically to determine an amplitude reflection coefficient.

Briefly, the amplitude reflection coefficient is a measure of the fraction of ultrasound that is reflected from an interface with unmatched acoustic impedances. Values

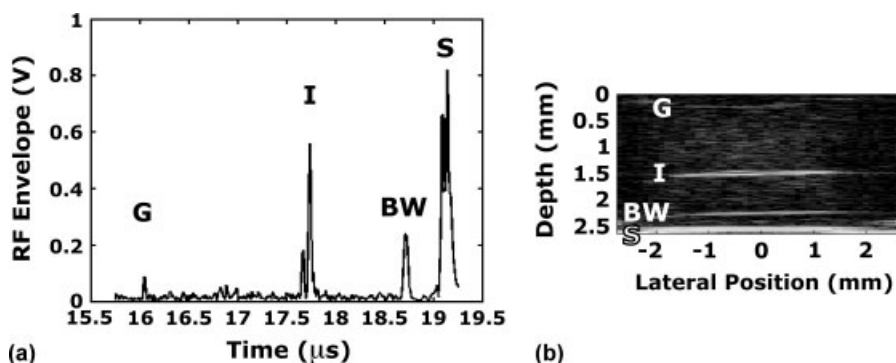


Figure 6. Single ultrasound signal (a) and B-scan image (b) from a two-layer, partially degrading construct with  $7.5 \times 10^7$  chondrocytes/ml in the hydrogel layer 1 day after being polymerized in apposition to an excised cartilage layer. Peaks in the ultrasonic signal correspond to the top surface of the gel layer (G), the interface (I) between the gel layer and the cartilage layer, the back wall (BW) of the cartilage, and the stainless steel plate (S) on which the two layer plate rested during measurement

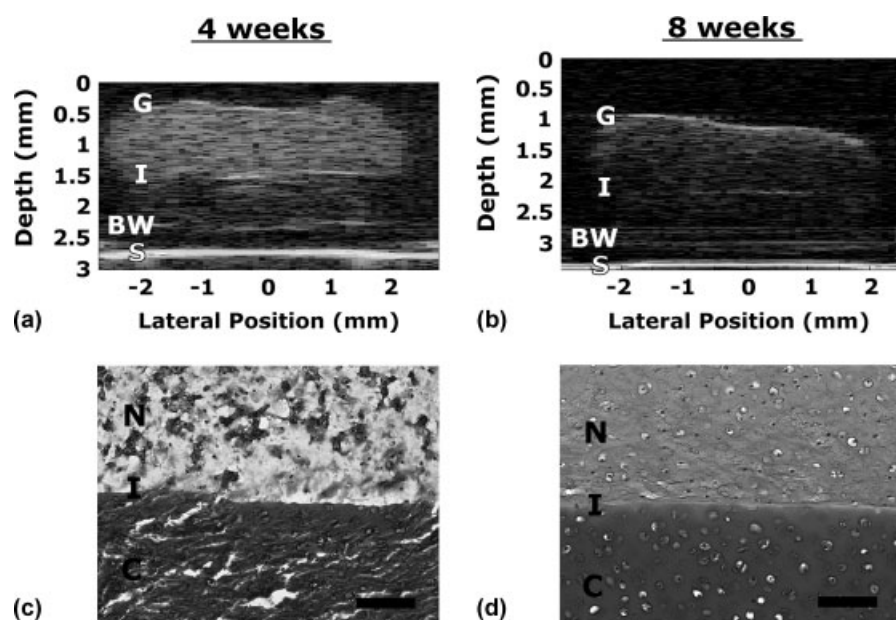


Figure 7. B-scan images (a, b) and safranin O/fast green histological staining (c, d, shown in greyscale) of two-layer, partially degrading constructs with encapsulated chondrocytes in the gel layer after 4 and 8 weeks of culture. Labels in the B-scan images correspond to the top surface of the gel layer (G), the interface (I) between the gel layer and the cartilage layer, the back wall (BW) of the cartilage, and the stainless steel plate (S) on which the two-layer construct rested during measurement. Additional labels in the histological images correspond to the gel–neotissue layer (N) and the native cartilage layer (C). Scale bars in the histological images =  $100 \mu\text{m}$

for the reflection coefficient were calculated with data from two-layer constructs and are shown in Figure 8 as a function of culture period and as a function of the average measured adhesive strength of corresponding three-layer constructs. While the reflection coefficients shown in Figure 8a generally decrease from the initial to the final time points for both conditions with cells, it is difficult to determine the significance of this change because a similar decrease was measured for two-layer constructs without encapsulated cells. Focusing on non-degrading gels with and without cells, the change in reflection coefficient with time was similar in these two-layer constructs, indicating that accumulation of secreted matrix at the interface does not significantly affect the calculated reflection. In addition, the data presented Figure 8b show that

the calculated reflection coefficient does not correlate with measured adhesive forces measured in three-layer constructs. The calculated reflection coefficients therefore do not appear to indicate adhesion or integration as they were calculated in this study. It is important to note that matrix evolution in the hydrogel layer was not accounted for in these calculations of reflection coefficient.

## 4. Discussion

### 4.1. Effects of controlled hydrogel degradation

Multi-layered constructs with cell–gel layers and native tissue layers were synthesized to investigate the

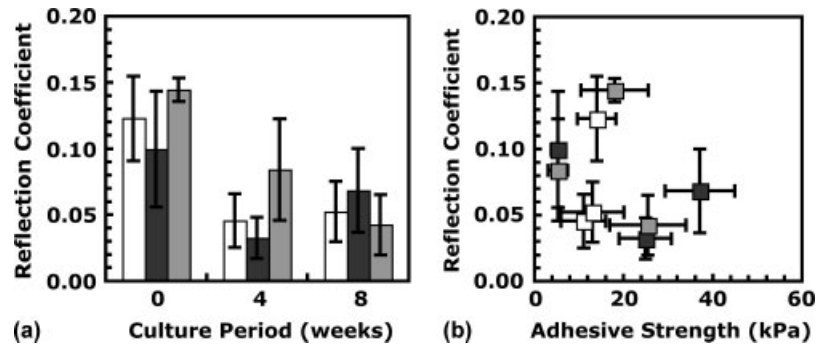


Figure 8. Comparison of the sample average reflection coefficients, calculated from the interface signal, with culture period (a), and with the average adhesive strengths for each experimental condition (b). Shading corresponds to non-degrading constructs without (white) or with (dark grey) encapsulated chondrocytes, and to partially degrading constructs with encapsulated chondrocytes (light grey)

integration of neocartilage generated in non-degrading or partially degrading PEG hydrogels with excised pieces of native cartilage tissue. Among the advantages of PEG hydrogels formed from the photopolymerization of divinyl macromonomers is the ability to incorporate block copolymers to make the gels hydrolytically degradable. Degradation is important because it enables chondrocytes to be encapsulated in gels that are mechanically robust, but gradually degrade to allow distribution of secreted ECM components. However, degradation was controlled in the gel layers of these constructs in order to prevent formation of major defects in the evolving cartilaginous matrix, and to maintain a minimal hydrogel network surrounding the encapsulated cells.

The non-degrading PEG gels applied in this study were specifically chosen to maintain proper cell morphology and prevent facile diffusion of the large cartilage ECM component, collagen, but at the same time allow distribution of smaller cell-secreted proteoglycans. Histological images confirmed that these gels controlled matrix diffusion as expected. In addition, the mechanical data for non-degrading constructs without cells confirmed that there was no significant change to the hydrogel network over the 8 week culture period. The increasing adhesive strength in non-degrading gels with encapsulated chondrocytes is therefore most likely related to the secretion and diffusion of proteoglycans. The difference between the initial adhesion strengths in the non-degrading conditions has to do with the presence or absence of cells in the gel. Encapsulation of chondrocytes at  $7.5 \times 10^7$  cells/ml introduces a heterogeneity to the macroscopic gel structure that results, in this case, in a decrease in measured adhesive strength.

Partially degrading co-polymer gels were used to provide a condition in which both collagen and proteoglycans could diffuse easily, at conditions previously found to maintain proper chondrocyte morphology and phenotype (Rice and Anseth, 2004). The initial decrease in measured adhesive strength from 0 weeks to 4 weeks was likely due to the significant amount of degradation that takes place in the crosslinked network in the hydrogel layers of these constructs, which also influences the accumulation

of matrix molecules. Since the partially degrading constructs should allow diffusion of both proteoglycans and collagen after such a significant amount of degradation, the increase in adhesive strength of these constructs from 4 to 8 weeks is likely due to secretion and reorganization of either or both collagen and proteoglycans.

Previous studies have shown that cell morphology is best maintained in non-degrading PEG gel formulations (Rice and Anseth, 2004), and it has long been known that chondrocyte morphology has an important impact on the isoform of collagen produced by the cells (Vondermark *et al.*, 1977). Current surgical techniques that utilize marrow stimulation tend to result in formation of scar tissue and good clinical results in the short term, but a number of long-term studies indicate that tissue regenerated following these procedures may not persist as an integrated, functional replacement tissue (Richardson *et al.*, 1999; Horas *et al.*, 2000; Hunziker, 2001). Thus, maintenance of cell morphology is likely important to the ability of chondrocytes to degrade and remodel ECM without forming a fibrocartilage layer between the native and repair tissue, and therefore may alter the rate or extent of degradation built into the hydrogel. For example, ratios of degrading to non-degrading macromonomer that appeared to be ideal for bulk matrix evolution may need to be adjusted to include higher amounts of non-degrading macromonomer to maintain a higher fraction of cells with a rounded morphology.

Some chondrocytes in the neotissue layers in Figure 3a–b appear large relative to those in the cartilage layer, potentially indicating hypertrophy. Interestingly, the same effect was not apparent in gels with a partially degrading gel layer, even following predigestion by collagenase type II. Previous studies of chondrocytes cultured in similar PEG hydrogels has shown production of type II collagen through 8 weeks of *in vitro* culture time (Rice and Anseth, 2004), which does not correspond to the matrix production that would be expected from hypertrophic chondrocytes. However, hypertrophy of cells at the interface would result in production of type X collagen and mineralization that could affect adhesion measurements and integration assessments. As such,

future studies should include some degree analysis of cells near the interfaces of neotissue with existing tissue.

#### 4.2. Effects of collagenase pretreatment

Another advantage of PEG hydrogels important to these studies is their ability to be formed *in situ* at a defect site. This advantage arises because the divinyl macromonomers can be dissolved in aqueous solution along with photoinitiator. This solution can be injected to fill any complex shape and be photopolymerized in apposition to the tissue being repaired. Since cartilage is mostly water, this was especially advantageous because we expected that macromonomer molecules would not only form mechanical interlocks by filling surface defects, but also form entanglements and a semi-interpenetrating network near the surface of the cartilage tissue. In the second group of studies described in this work, diffusion of large molecules into the cartilage surface was improved by decreasing the density of the crosslinked collagen network at the surface of the cartilage matrix prior to photopolymerization. The results presented do not support the assertion of interpenetration of macromonomer molecules, because mechanical measurements of adhesion are similar at the early time point. However, the predigestion step resulted in higher measured adhesion strengths after 8 weeks of *in vitro* culture, and also appeared to have a positive effect on cell morphology and behaviour. It is likely that the surface treatment made it easier for cell-secreted collagen to diffuse into the native tissue, and it also appears that the treatment reduced the likelihood of cells to interact in an adhesive manner. The latter may be the result of a decrease in number or effectiveness of cell attachment sites on the cartilage surface as a result of the pretreatment with collagenase.

#### 4.3. Non-destructive analysis

The details of measuring and describing integration are important to note. In animal models, as well as studies performed *in vitro*, histological analysis is very important. Histology can provide simple qualitative evidence of integrative repair by showing homogeneity of ECM across an interface and also by showing the disappearance of an interface over time. In addition, adhesive strengths can be quantitatively measured by a variety of mechanical testing techniques that range from simple tension testing to more sophisticated push-out tests (Reindel *et al.*, 1995; Peretti *et al.*, 1999; Bravenboer *et al.*, 2004; Moretti *et al.*, 2005). Mechanical testing is always presented alongside histological images, as the adhesive strengths are not necessarily due to tissue integration. This combination of evidence is especially important in cases where the initial adhesion has been modified, as mentioned above. To complement these approaches, alternative measurement techniques that could be used to non-destructively assess

the continuity of the interfacial region would have utility in improving our understanding of tissue integration. Recently, an MRI technique has been utilized to non-destructively assess tissue–hydrogel integration resulting from different chemical- or tissue-initiated gel syntheses (Ramaswamy *et al.*, 2006). A different non-destructive measurement may be made through ultrasound analysis.

Ultrasound was initiated by a 50 MHz transducer, and the ultrasound signal travelled down through a buffer solution and the construct to a stainless steel plate on which each sample was placed during testing. While B-scans were helpful to qualitatively assess construct shape and general matrix accumulation, the ultrasound signals were analysed quantitatively to calculate a reflection coefficient from the interface between native tissue and the cell–gel construct. The change in reflection from the interfaces of constructs without cells may be related to a decrease in density near the excised cartilage surface as proteoglycans near the interface slowly diffuse out of the native tissue into the gel, or to accumulation of media proteins that takes place near the interface in the gel layer. One would expect such effects to be small and additive with respect to the accumulation of cell-secreted matrix proteins at the interface, but the conditions with or without cells have similar relative values of the reflection coefficient at all measurement times. It should be noted that ultrasound analysis did, unfortunately, contribute to the complexity of the present study, specifically with ultrasound analysis times that were inconsistent with other measurement times due to limited availability of the instrument.

The only relative change among experimental groups shown in Figure 8a is related to the partially degrading constructs from week 4 to week 8. It makes intuitive sense that the partially degrading gel portion of these constructs at 4 weeks may be significantly degraded, decreasing the acoustic impedance of the gel layer and the adhesion of that gel layer to the native tissue and resulting in increased reflection of ultrasound from the interface. As matrix molecules in the partially degrading gel diffuse to form homogeneous neotissue, the density would increase throughout the gel layer and likely form a better impedance match with the cartilage layer, although this would not necessarily be indicative of a better mechanical and histological outcome. It is also important to note that the changes to attenuation and speed of sound in the evolving neotissue layer were not accounted for in the calculations of reflection coefficient and may have more importance than initially expected. Unfortunately, the amplitude reflection coefficient calculated for these developing constructs does not appear to provide a quantitative assessment of integration. However, the reflection calculation is more straightforward when the top interface of a material is compared with an aqueous buffer solution, and is therefore useful for characterizing treatments of soft tissue surfaces in single-layer samples. This technique will require further development of ultrasound analysis of bulk matrix accumulation in order

to be applied quantitatively in the analysis of this important interface.

Ideally, the degradation of a hydrogel construct will be controlled in order to produce homogeneous tissue in the bulk that integrates well with surrounding tissue and that can be examined by a non-destructive analysis technique. The sensitivity of cells at interfaces of cell-laden constructs with native tissue is not a trivial problem in the design of future generations of biomaterials for tissue engineering applications. Simple enzymatic tissue pretreatments provide clues to the importance of the interactions between these biomaterials and existing tissues, and the development of non-destructive analysis techniques will allow these clues to become apparent in shorter experimental time periods by allowing collection of data at higher frequency with the same number of constructs.

## 5. Conclusions

The effects of gel degradation and collagenase predigestion of excised cartilage surfaces on the integration of engineered tissue with excised cartilage disks were examined. Chondrocytes encapsulated in a non-degrading gel layer were more likely to maintain their preferred, rounded cell morphology. Histological and mechanical evidence suggested that these cells and their secreted proteoglycans were interacting with the native tissue surface to improve adhesion and integration of the initial three-layer construct. While collagen secreted by encapsulated cells would intuitively be critical to this integration, this large molecule appeared to have a major effect on the measured adhesive strength only when the native tissue layer was pretreated with collagenase to open up the native collagen network prior to polymerization of the hydrogel layer. Ultrasound was shown to be an effective tool for the non-destructive visualization of these interfaces. However, analysis of the amplitude reflection coefficient did not provide a quantitative measure of integration by the analysis methods described in this work.

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