Evaluation of Fracture Toughness for Articular Cartilage and Hydrogels in Cartilage Tissue Engineering

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ABSTRACT

Recently, biomaterials-based tissue engineering strategies such as hydrogels have offered great promise in repairing articular cartilage. Mechanical failure testing in outcome analyses is given the crucial clinical importance to the success of engineered constructs. Interpenetrating networks (IPNs) are gaining more attention due to their superior *mechanical* integrity. Extensive fracture toughness (here refers to the apparent fracture toughness) work has been performed on articular cartilage but seldom performed on regenerative biomaterials such as hydrogels. The objective of this study was to provide a combination testing method of apparent fracture toughness applied to both articular cartilage and hydrogels in cartilage tissue engineering. In this study, apparent fracture toughness of three groups was evaluated: acellular hydrogels, cellular hydrogels, and articular cartilage based on the modified single-edged notch test and American Society for Testing and Materials (ASTM) standards on the single-edged notch test and compact tension test. The results obtained in this thesis demonstrated that the toughness of articular cartilage (348 ± 43 MPa*mm^{½}) was far more than that of hydrogels. 6K molecular weight (MW) 20% acellular IPNs look promising with a toughness value of 10.8 ± 1.4 MPa*mm^{½}, which was the highest among the hydrogel groups. This method preserved the integrity of the articular cartilage and the consistency of each specimen to obtain the data as accurate as possible. Although geometry limitations existed, a new method was developed to evaluate hydrogels and cartilage in a manner to enable a more relevant direct comparison for fracture testing of hydrogels for cartilage tissue engineering.

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CHAPTER 1: INTRODUCTION

Injuries to articular cartilage cause major problems and have become the main reason that arthritis is the leading cause of disability in the United States today (1). Biomaterials-based tissue engineering strategies offer great promise, including the use of hydrogels to regenerate articular cartilage (2-5). The overall objective of this thesis was to evaluate toughness of articular cartilage and hydrogels in cartilage tissue engineering.

Toughness reflects how much energy the material will absorb to fracture (6) and the ability to withstand crack propagation ultimately determines the toughness (7). However, virtually all of the hydrogel studies to date in articular cartilage tissue engineering have lacked evaluation of apparent fracture toughness (2). Some studies only consider ultimate stress or strain, which may suffer with regard to reproducibility (6, 8).

When evaluating the toughness of hydrogels within articular cartilage regeneration, there are few studies involved. For example, Dekosky *et al.* (5) compared the toughness between IPN and single network hydrogels by applying compressive loading. However, many of the toughness studies on hydrogels are outside of the articular cartilage tissue engineering community (8-10). Meanwhile, toughness on articular cartilage is gaining more and more attention (7, 9, 11-14). In chapter 2, the literature was reviewed on the toughness measurement of hydrogels and articular cartilage so as to identify the common ground for these distinct fields.

More specifically, how to best identify the fracture mechanics methods most suitable for evaluating both hydrogels and cartilage was sought.

To achieve the overall objective, based on the apparent fracture toughness measurements of articular cartilage and of hydrogels outside of cartilage tissue engineering, the modified single edge notch test with ASTM standards was revised to establish the groundwork for linking methodologies between fracture testing of cartilage and hydrogels, which will be demonstrated in detail in chapter 3. In chapter 4, 6K 20% acellular hydrogels from five different batches were tested using the same methodology in Chapter 3 to confirm reproducibility of synthesis and to provide an adequate sample size to detect statistical significance. In an effort to provide recommendations for evaluation of fracture properties for hydrogels in cartilage tissue engineering, chapter 5 presents the concluding thoughts on limitations as well as my recommendations for future directions for this line of work.

CHAPTER 2: A Review of Evaluation of mechanical toughness for hydrogels in cartilage tissue engineering

ABSTRACT

Injuries to articular cartilage are a major problem, being one of the main reasons that arthritis is the leading cause of disability in the United States. Unfortunately, cartilage repair strategies are notoriously unreliable and/or complex. Biomaterials-based tissue engineering strategies offer great promise, including the use of hydrogels to regenerate articular cartilage. Mechanical integrity is arguably the most important functional outcome of engineered cartilage, although mechanical testing of hydrogel-based constructs to date has focused primarily on *deformation* rather than *failure* properties. As the field of cartilage tissue engineering matures, this community will benefit from the inclusion of mechanical failure testing in outcome analyses, given the crucial clinical importance to the success of engineered constructs. However, there is a tremendous disparity in the methods for evaluating mechanical failure of hydrogels and articular cartilage. In an effort to bridge the gap in mechanical testing methods of articular cartilage and hydrogels in cartilage regeneration, this review classifies the different toughness measurements for each. In comparing these toughness measurement methods, it appears that the best option for evaluating mechanical failure of hydrogel-based constructs for cartilage tissue engineering may be tensile testing based on the single edge notch test, in part because specimen preparation is more straightforward and a related ASTM standard can be adopted.

INTRODUCTION

Injuries to articular cartilage are a major problem, being one of the main reasons that arthritis is the leading cause of disability in the United States (1). Biomaterials-based tissue engineering strategies offer great promise, including the use of hydrogels to regenerate articular cartilage (2-4). Improving mechanical integrity of hydrogel-based constructs is of great importance to cartilage regeneration.

Hydrogels have been investigated for use in a variety of biomedical applications such as tissue engineering (4, 5, 15) and drug delivery (16, 17). To replace damaged cartilage tissue, hydrogels will be required to provide deformation properties and resistance to fracture. The most common evaluation of deformation properties is through modulus measurement (2, 4, 5, 18), whereas the resistance to fracture (i.e., failure) can be evaluated by apparent fracture toughness. Apparent fracture toughness reflects how much energy the material will absorb to fracture (6) and governs the response of given materials to crack propagation (7). However, virtually all of the hydrogel studies to date in articular cartilage tissue engineering have lacked evaluation of apparent fracture toughness (2). Some studies only consider ultimate stress or strain, which may suffer with reproducibility (6, 8).

Thus to establish testing methods to evaluate hydrogels, it is necessary to look into apparent fracture toughness studies with articular cartilage as well as apparent fracture toughness studies from outside of the cartilage tissue engineering field (9, 10, 19-22). Ultimately, to have an effective hydrogel for cartilage tissue engineering, both the deformation properties and fracture properties should match

those of articular cartilage. Therefore, established methods to test cartilage fracture properties can be used as a guide in testing hydrogels for cartilage tissue engineering These tests include the single edge notch test, trouser tear test, indentation test, etc. (23, 24).

Based on the apparent fracture toughness measurements of articular cartilage and of hydrogels outside of cartilage tissue engineering, we will establish the groundwork for linking methodologies between fracture testing of cartilage and hydrogels in an effort to provide recommendations for evaluation of fracture properties for hydrogels in cartilage tissue engineering (Fig. 2.1).

TOUGHNESS MEASUREMENT OF ARTICULAR CARTILAGE

The fracture behavior of articular cartilage is intrinsically connected to its structure (25). The articular cartilage was divided into four macroscopic layers (26, 27). The surface layer, named the superficial zone, is known to be more resistant to shear stress and wear than the underlying layers due to the horizontal orientation of the collagen fibers (28). Below this lies the middle and deep zones in which fibers turn obliquely to form a radially aligned 3-D mesh (25). The underlying calcified zone bears the compressive load (25, 29) since collagen fibers distribute load perpendicular to the surface of articular cartilage.

Since articular cartilage tissue is anisotropic, crack propagation can vary, making it difficult to obtain consistent apparent fracture toughness measurements. Even in isotropic, linearly elastic materials, different loading modes result in

distinctly different values for apparent fracture toughness (11, 24). Therefore, rather than evaluating apparent fracture toughness under compression, where reproducibility can be a challenge, we turn our attention to other trusted methods of apparent fracture toughness measurement. In this context, there are three primary fracture loading modes, which are concisely explained as follows. Mode I loading opens a crack by inducing tensile stresses normal to the crack plane (Fig. 2.2A). In contrast, Mode II loading propagates a crack between two surfaces by inducing in-plane shearing loads (Fig. 2.2B). Finally, Mode III loading extends a crack by transverse (out of plane) shearing (Fig. 2.2C). Compared to Mode II, Mode I and Mode III are more commonly used for cartilage because these modes test tensile stresses and tear, respectively, which have been the preferred modes of testing in the cartilage biomechanics community (24).

We are certainly cognizant of the fact that cartilage failure as a biological phenomenon in osteoarthritis is typically considered in the context of an impact injury followed by a cascade of signaling events over an extended period of time that result in the breakdown of cartilage structure and thus the loss of mechanical integrity. However, for the purposes of this review, we examine cartilage as a material, and thus review studies that have evaluated its failure properties as a material, which will serve to facilitate the juxtaposition of cartilage failure and hydrogel failure. Therefore, the following sub-sections will discuss loading Mode I (opening mode) and Mode III (out of plane mode and indentation test). Based on the results of investigations of cartilage failure with these different mechanisms, we will conclude

this section with suggestions for selecting a reliable method for toughness measurement of articular cartilage in the context of looking forward tissue engineering studies.

Mode I – Modified single edge notch test, and single edge notch test

Based on Mode I loading, Chin-Purcell and Lewis (30) initiated the modified single edge notch test (MSEN, Fig. 2.3) by propagating the crack from the subchondral bone of the adult mongrel canine patella into the deep and middle zones of articular cartilage. They equilibrated the sliced specimens for roughly 1 hour in a temperature-controlled saline bath at 37 ℃. After equilibration, they placed each specimen in specially designed holders to grip the bone section. The grips were spring loaded with the same spring tension for each test. In this way, *the grips grabbed the subchondral bone instead of the articular cartilage* in the normal Mode I loading test, which helped to avoid slippage and deformation of the articular cartilage. Adams *et al.* (12) supplemented the above research by finding that the thickness (between 0.7 mm and 2.7 mm) of the cartilage samples used in the MSEN test had no effect on the apparent fracture toughness.

Stok and Oloyede (7, 25) supplemented these aforementioned studies by testing all four cartilage zones, instead of only the deep zone, in the single edge notch test. They shaved the cartilage from the bovine bone and trimmed the cartilage into strips. A notch was made on one edge of the cartilage and the tensile loading was applied at both ends of the cartilage (Fig. 2.4). Furthermore, they analyzed crack

propagation at varying rates of tensile loading (1.5, 3.0 and 4.5 mm/min) and found that the stress measured during crack propagation did not vary significantly with the different loading rates. Therefore, they proposed that it was the structural variations between the diverse zones of the tissue, rather than the speed of loading, that predominately determined the characteristics of fracture in articular cartilage (7).

The main difference between the modified single edge notch test and single edge notch test, both classified as Mode I tests, is the geometry of the samples. Specifically, in the modified single edge notch test, the cartilage remains attached to the bone and this single osteochondral unit is sectioned into slices as whole pieces, whereas in the single edge notch test, the cartilage is removed from the bone. This difference in the geometry affects the shape factor in the data analyzing model, which will thus influence the final values obtained.

Mode III – Trouser tear test

Mode III loading was originally derived from anticlastic plate bending (ACPB) (31). Just as its name implies, ACPB (32) was defined as a rectangular plate undergoing a twisting type of load to deform by two opposite curvatures wherein the plate assumes a saddle-shaped configuration (Fig. 2.5). Another name for ACPB loading mode is the plate twist method.

As for testing articular cartilage, Chin-Purcell and Lewis (30) introduced the trouser tear test based on the mechanics of Mode III loading. The procedure they used was to cut up the middle of the cartilage, dividing it into two pieces as “trouser legs”

(Displayed schematically in Fig. 2.6) with a scalpel through the bone section to the cartilage base. The trouser legs were approximately 1.5 mm wide. The bone on the legs was carefully placed into the grips so that the length of the leg was parallel to the line of loading. The loading rate was the same as the MSEN test, tearing the cartilage apart along the radial direction of the cartilage. The tear always progressed along this direction. The critical load was determined from the load displacement curve as the first maximum load on the typical curve. Based on the fact that viscous dissipation would not affect the data reduction; energy supplied or dissipated in the cartilage tests was determined by the authors to be negligible compared to the tearing energy (30). Therefore, it was assumed there was no viscous dissipation in the original derivation.

Mode III - Micro-penetration test

The indentation test provides a method of determining how much compressive load articular cartilage can withstand. Most of the models of the indentation technique focus their attention on mechanical characteristics of articular cartilage such as surface roughness, wear and elastic modulus *in situ* and *in vivo* (33-38). When combined with atomic force microscopy (AFM), SEM, etc., we can obtain a much more explicit view of those properties of articular cartilage (23, 39, 40). Though fracture failure properties of cartilage are essential in injury repair and disease, few indentation methods exist for measuring them.

Simha *et al.* (13) introduced a new method of indentation testing for measuring the apparent fracture toughness of articular cartilage in 2004. A

penetration or fracture defect in the surface of intact cartilage, which was previously attached to underlying subchondral bone, was created by a small conical tip (Fig. 2.7). Unlike tensile and conventional fracture tests, preparation of small or regularly shaped specimens is not required with this method, which is an advantage because this type of specimen preparation is difficult in small animals due to the small volume of cartilage tissue. Due to the small indenting tips they used, the indentation depths were shortened to the order of 100 μm, which were greatly larger than those in conventional Nano-indentation methods. Therefore, the name of the described methods above was designated "micro-penetration" by Simha *et al.* (13).

To identify whether penetration had occurred, they stained the specimen with India ink after testing the first group and examined it under an optical dissecting microscope to identify the penetration by localization of India ink in the created defect. Then apparent fracture toughness measurement followed. The apparent fracture toughness, T, was calculated as

$$T = \frac{W_p \sqrt{2}(1 + \cos\alpha)^{3/2}}{\pi h_{pen}^{\ 2} \sin\alpha} \quad \ldots\ldots (1)$$

$$Penetration\ work = W_p = \int F\ dh_s \ \ldots\ldots (2)$$

where W_p was the penetration work, F was the indenting force, h_{pen} was the total penetration depth, h_s was the displacement during penetration and α was the apex angle of the cone.

Summary

All of the above methods are tabulated in detail (Table 2.1), including their advantages and disadvantages. Among those methods, the modified single edge notch test (a Mode I test) is promising in future studies because it is easy to manipulate and visualize. It can also rely on the ASTM standards designed for the singe edge notch test. In contrast, during the single edge notch test, the sample may be easily over-gripped, which can affect the measurement. The tear test is limited in that it is difficult to observe the whole measurement process. The indentation test may be promising, although additional studies would be required to further support its use.

TOUGHNESS MEASUREMENT OF HYDROGELS

Hydrogels enable encapsulation of cells and affect their gene expression (9) under physiological conditions because of their bio-amenable properties such as their high water content capacity, mild gelation conditions for abundant naturally occurring polymers and their response under loading (41). For scaffolds in articular cartilage regeneration, the fracture properties of synthetic hydrogels are particularly critical, since natural tissue requires a mechanical integrity that can sustain large deformations without fracture (42-44).

In the following sub-sections, we will introduce the methods that have been used to evaluate the toughness of hydrogels in general. However, in an effort to determine a reliable method for toughness measurement of hydrogels in cartilage regeneration, we will discuss only the methods that can be applied to both hydrogels and cartilage.

Tensile test: with and without notch

The tensile test is set up to measure resistance to tensile loading. For testing hydrogels, there are mainly two different methods. One, "with notch," is based on the opening Mode I – single edge notch test, analogous to what was discussed previously with cartilage. The other, "without notch," is pure tensile testing.

Kong *et al.* (9) investigated various aspects of gel cross-linking to independently regulate the elastic modulus (E) and toughness (W_0). After inventing a new type of alginate gel, they assessed the toughness of these hydrogels using the single edge notch test (i.e., tensile test with notch). They introduced a notch in the rectangular gel strips (10 ×3 ×0.1 cm) with a razor blade. The strips were extended at a constant deformation rate of 1 mm/min with initial notch length varying from 1 to 3 mm, and stresses were measured. The total work to fracture (W_t) was calculated from the area of stress vs displacement curve. W_0 was evaluated from the slope of W_t vs. the width of sample between the two initial notches.

Smith *et al.* (8) studied the toughness of hydrogels, both in air and in phosphate-buffered saline at different temperatures, by conducting failure tests and comparing the elastic modulus and toughness of the specimens. In the test, dog-bone specimens (laser-cut according to dimensions specified in ASTM D 638-03 Type IV or V) were loaded on a universal testing machine (MTS Systems, Insight 2) using a 2 kN load cell with a 1 mm/min strain rate. The elastic modulus was calculated as the slope of the initial linear region of the stress–strain curve, while toughness was

calculated as the area under the stress–strain curve up to the fracture stress point in units of MJ/m^3. They concluded that the primary factors that influenced the toughness of hydrogels were the test temperature relative to the glass transition temperature, the water content and the network structure.

Mode III-Tear test

Tanaka *et al.* (10) measured the toughness of PAMPS/PAAm double network gels with different cross-linking densities. They cut the gels into the standardized rectangular shape (30 mm width) by a gel cutting machine (Dumb Bell Co., Ltd.) (Fig. 2.8A). The notch length was 20 mm, and the two arms of the test sample (Fig. 2.8B) were placed in the grips. During the test, only the upper grip was pulled upward at a constant velocity V_p. By recording the tearing force F, they calculated fracture energy G using the following equation,

$$G = \frac{F_{ave}}{2w} \ldots\ldots (3)$$

where F_{ave} is the average of F during tear and w is the width of the gels.

They assumed there was no elongation of the arms and the crack velocity V was equal to $V_p/2$ without taking account of the change of elastic energy stored in the pulled arms. Even if taking elongation of the arms into account, the tearing velocity V was changed from 0.5×10^{-5} to 0.5×10^{-2} m/s which is negligible. Besides, the change of elastic energy stored in the pulled arms resulted in only a few percent of correction for G and V, which is insignificant too.

Compression test

The most straightforward evaluation of the toughness for hydrogels is through the compression test, through which the elastic modulus and shear modulus can also be obtained. Dekosky *et al.* (5) were the first to develop a new method for encapsulating cells in interpenetrating network (IPN) hydrogels of superior mechanical performance. They used dynamic mechanical analysis (DMA) to gain the mechanical performance of a new IPN hydrogel based on two biocompatible materials -- agarose and poly(ethylene glycol) diacrylate (PEG-DA). During their tests, all of the hydrogel samples were cut into circular shapes and placed on to the compression plates, which were lubricated with mineral oil. Toughness was calculated by numerical integration of the stress-strain curve generated by compressing each sample at a rate of 0.0005 mm/s.

Summary

Hydrogel apparent fracture toughness tests have included tensile tests both with notch (Mode I) and without notch, in addition to the tear test (Mode III) and compression. All the above methods are tabulated in detail (See Table 2.2) and their advantages and disadvantages are provided. Referring to the table, the single edge notch test based on the tensile testing for polymers from ASTM standards is promising because different types of fracture energy can be calculated such as work to fracture dissipated outside the process zone and the essential work at the process zone. Other tests have limitations that may show some unsatisfied aspects. For

example, tensile test without notch can only evaluate the total apparent fracture toughness. The tear test has difficulties in loading the samples and large amount of sample materials may be needed to meet the standard geometry. Besides, there are many ASTM standards related to composite polymers and plastic materials but no specific standards for hydrogels. However, for hydrogels in cartilage regeneration, there are no specific standards for toughness measurement.

DISCUSSION

In a general view of testing articular cartilage, though there are several different kinds of methods for testing the apparent fracture toughness value, diverse cartilage sources may affect the choice of testing method. For example, in the single edge notch test, articular cartilage from small animals may have limited length for the grippes to grab. With the articular cartilage from the ankles of even large animals, the trouser tear test (Mode III) cannot be chosen because the thinness of the cartilage layer and its irregular surface will make it difficult to section into the standard geometry.

Another point of consideration is that cartilage is anisotropic, which means that even if samples from the same source of articular cartilage were to be tested, different toughness values may be obtained when using different testing methods since each method depends on the unique qualities of the specimen. For example, in Chin-Purcell and Lewis's work, they sectioned off the superficial zone of the cartilage to avoid the aberrant crack propagation and only tested the fracture

resistance in the deep and middle zones. In contrast, in their micro-penetration test, the fracture resistance was measured in the surface.

Furthermore, variations in toughness values may occur even with the same testing method for cartilage. Few papers actually mentioned how the crack position was verified. Finding a method to best ensure that cracks are made consistently may help to overcome the problem. Though a number of investigators have supplemented Chin-Purcell and Lewis's work in testing apparent fracture toughness in tension, future research still remains such as visualizing the crack propagation process during the MSEN test so as to identify the fracture resistance in each typical zone.

Of course the isotropic structure of hydrogels enables us to make any size we want to fit into different loading modes. Thus, different testing methods can be applied such as the tensile test, tear test or compression test, to one type of hydrogel. Strict attention is necessary to avoid making any micro-cracks when loading those hydrogels into the testing machine, especially when using the trouser tear test or single edge notch test.

However, based on the testing methods of articular cartilage, we may narrow those methods for hydrogels down to fit the purpose of evaluating mechanical failure of hydrogel-based constructs for cartilage tissue engineering. In comparing the articular cartilage and hydrogel-based constructs side by side, the difference in testing conditions such as geometry factor and in testing method would ideally be eliminated. Thus, though the micro-penetration method works well on articular cartilage with easy manipulation, it may be limited in testing hydrogels.

To consolidate the two distinct fields of cartilage and hydrogel fracture testing, the best method for both cartilage and hydrogels may be tensile testing based on Mode I because the geometry limitation of cartilage may fail in trouser tear test or compression test. Though there is no geometry limitation of hydrogels in applying the trouser tear test or compression test, the data may be difficult to reproduce because the hydrogels in cartilage regeneration are normally softer to make cracks consistently compared to other types such as contact lenses (21, 45-47). Thus, the test in Mode I may be the most appropriate approach for testing both hydrogels and articular cartilage and thus allowing for more relevant comparisons. Besides, Mode I has the solid data analytical method on the ASTM standards.

CONCLUSION

Biomaterials-based tissue engineering strategies offer great promise, including the use of hydrogels to regenerate articular cartilage. Cartilage and hydrogels have different fracture mechanics, and because of these differences cartilage tissue engineering research community would benefit from the development of a uniform method that can be applied to both materials. Based on fracture mechanics literature from both the cartilage and hydrogel fields, a leading candidate for a toughness testing method for hydrogels in cartilage regeneration may be the modified single edge notch test. Providing standards and testing methods that accommodate for both hydrogel and cartilage will allow us to improve the failure properties of hydrogels and will ultimately lead to better tissue replacements for damaged articular cartilage.

Chapter 3 Evaluation of Apparent Fracture Toughness for Articular Cartilage and Hydrogels in Cartilage Tissue Engineering

ABSTRACT

Recently, biomaterials-based tissue engineering strategies, including the use of hydrogels, have offered great promise in repairing articular cartilage. Mechanical failure testing in outcome analyses is of crucial clinical importance to the success of engineered constructs. Interpenetrating networks (IPNs) are gaining more attention due to their superior *mechanical* integrity. This study provided a combination testing method of apparent fracture toughness both applied to articular cartilage and hydrogels. Apparent fracture toughness of three groups – acellular hydrogels, cellular hydrogels and articular cartilage – were evaluated based on the modified single edge notch test and ASTM standards on single edge notch test and compact tension test. The results demonstrated that the toughness for articular cartilage (348 ± 43 $MPa \cdot mm^{½}$) was much higher than that for hydrogels. Molecular weight (MW) 6K 20% acellular IPNs looked promising with a toughness value of 10.8 ± 1.4 $MPa \cdot mm^{½}$, which was the highest among the hydrogel groups. In addition, higher molecular weights of poly (ethylene glycol) diacrylate gels may increase the apparent fracture toughness and higher PEG concentration may increase the toughness as well. Though some geometry limitations exist, a new method was developed to evaluate hydrogels and cartilage in a manner that enabled a more relevant direct comparison for fracture testing of hydrogels for cartilage tissue engineering.

INTRODUCTION

Injury to articular cartilage is one of the main reasons that arthritis is the leading cause of disability in the United States (1). However, using hydrogels in biomaterials-based tissue engineering to regenerate articular cartilage offers great promise (2-5, 22). Improving mechanical integrity, including both the *deformation* and *failure* of hydrogel-based constructs, is of great importance to cartilage regeneration. Although mechanical failure testing in outcome analyses is of crucial clinical importance regarding the success of engineered constructs, mechanical testing of hydrogel-based constructs to date has focused primarily on *deformation* rather than *failure* properties (48-53).

Failure properties can be characterized by apparent fracture toughness, which reflects how much energy the material must absorb to fracture (6) and governs the response of given materials to crack propagation (7). There exist various methods to test apparent fracture toughness in metals and plastic films such as the single edge notch test, trouser tear test, indentation test, etc. Due to a lack of studies on failure properties of hydrogel-based constructs, the overall objective of this paper is to develop a method to evaluate apparent fracture toughness for both articular cartilage and hydrogels in cartilage tissue engineering. Ultimately, the goal is to create an effective hydrogel for cartilage tissue engineering, and ideally both the deformation properties should match those of articular cartilage, and the fracture properties should meet or exceed those of articular cartilage.

Among those apparent fracture toughness tests, the modified single edge notch test is preferred for application to both hydrogels and articular cartilage because it can avoid overstressed factors induced by grips and it is derived from the single edge notch test, which has solid fundamental models to analyze data. Chin-Purcell and Lewis (30) first invented the modified single edge notch test to measure the toughness of bovine patellar cartilage based on the work of Mai and Atkins (54) and Srawley and Gross (55). Adams *et al.* (12) supplemented their work by suggesting that the thickness of the specimen did not affect apparent fracture toughness. However, the models for analyzing apparent fracture toughness data were geometry dependent. Thus, after reviewing related ASTM standards, we adopted ASTM D 5045 – 99 (2007) to evaluate the apparent fracture toughness for both articular cartilage and hydrogels in cartilage tissue engineering.

Based on this apparent fracture toughness method, we will establish the groundwork for linking methodologies between fracture testing of cartilage and hydrogels in an effort to evaluate fracture properties for hydrogels in cartilage tissue engineering. Our hypothesis was that IPNs would be significantly tougher than PEGDA for a range of PEG concentrations and molecular weights.

MATERIALS AND METHODS

Specimen preparation

Cartilage specimens:

Hog ankles (n=8, which were all from males) from either York or Barron, from 5 months to 7 months old and weighing from 97 to 195 kg were obtained from a

local slaughterhouse. The ankles were carefully opened within 24 h of the hogs' death. Then upper joint compartments were cut off (Fig. 3.1), wrapped in Kim Wipes, soaked in PBS (0.01 M phosphate buffered saline – 0.138 M sodium chloride, 0.0027 M potassium chloride) and stored at -20 °C. Cartilage specimens (thickness from 0.901 to 1.185 mm) (Fig. 3.2) were sectioned from the central portion of each ankle of the ten different hogs by bone saw. Thus the sample size for the articular cartilage was n=10.

Acellular hydrogel specimens:

2 – Hydroxyethyl agarose (Type VII) was obtained from Sigma- Aldrich. Two different molecular weights (2000 Da and 6000 Da) of PEG diacrylates (DAs) were obtained from SunBio (Anyang city, South Korea). Photoinitiator Irgacure 2959 (I-2959) was purchased from Ciba. 0.2g agarose powder was added to 10 ml PBS and autoclaved for 30 min to yield a 2% w/v agarose solution. When the agarose had cooled to 39°C, it was pipetted into rectangular silicon rubber molds (10 mm width, 20 mm length, 1 mm height) between glass plates. After 10 min cooling at 4°C, the gels were removed and added to a reservoir of PBS for equilibrating at least 24 h before synthesizing PEGDA and IPNs. A solution of 0.1% w/v I-2959 photoinitiator in deionized (DI) water was dissolved separately in both 15% w/v and 20% w/v 2K molecular weight (MW) of solution PEGDA in PBS at room temperature. One rectangular agarose gel was cut into eight pieces and added for each mL of monomer solution to soak under constant agitation using a rocker for 2.5 h. Then four pieces of

gel were placed in one rectangular silicon mold as before between optical glass microscope slides, and the surrounding space was filled with excess PEGDA/ PBS solution from the soak vials. The gels were exposed to ultraviolet light for 5 min on each side using 312 nm light, 3.0 mW/cm^2 (XL-1000; Spectronics Corp.). Acellular gel samples were then cut from both the acellular PEG-DA area and the IPN area by razor blades and added to excess PBS. The same procedure was applied on the synthesizing 6K MW 15% and 20% PEGDA and IPNs. All the gels were allowed to equilibrate in PBS for at least 24 h before testing.

Encapsulating cells within hydrogel specimens:

Rabbit chondrocytes were thawed and resuspended in a culture medium and plated in monolayer for expansion. Incubation was maintained at 37 °C with 5% CO_2, and fresh culture medium was provided every 48 h. The culture medium consisted of Dulbecco's modified Eagle's medium with 4.5g/L D-glucose supplemented with 10% FBS, 1% nonessential amino acids, 50 μg/mL ascorbic acid, and 0.25 μg/mL penicillin-streptomycin fungicide. The cells were expanded until 80% - 90% confluence was reached, at which point they were detached with 1× trypsin-ethylenediaminetetraacetic acid and labeled as passage 1. The medium and supplements were obtained from Invitrogen.

Chondrocytes were resuspended in PBS at a high concentration, while an agarose solution was prepared by adding 0.3 g agarose powder to 10mL of PBS and autoclaved for 30 min. Agarose solution temperature was monitored under aseptic

conditions until 39°C was reached, at which point the chondrocyte suspension was added to the molten agarose in a 1:2 ratio to form two types of cell suspension solution – concentration of 10 million cells/mL and 5 million cells/mL in 2% agarose. The cell suspension was then pipetted into sterilized silicon molds to form rectangular constructs (10 mm width, 20 mm length, 1 mm height). The molds were cooled at 4°C for 10 min, and then each type of the constructs were removed and added to a petri dish (Fisher, 15 mm in height). Each dish was supplied with 25 mL of fresh growth medium, and placed in a sterile incubation environment at 37°C for 24 h. Afterward, constructs were soaked separately in a sterile-filtered 15% or 20% w/v solution of 2K PEGDA and PBS with 0.1% I-2959 photoinitiator for 2.5 h (6 h of soaking was allowed for 6K PEGDA). An incubated orbital shaker was used to allow PEGDA to diffuse into the agarose–cell construct. For PEGDA crosslinking, gels were placed into a sterile rectangular silicon chamber between two optical glass microscope slides and surrounded with excess sterile PEGDA/I-2959 soak solution. With a 312 nm wavelength light at 3.0 mW/cm^2 intensity, the PEGDA network was polymerized for 5 min on each side (10 min total). Using a razor blade, rectangular gel samples were then cut from the center of the IPN area. Both PEGDA and IPN gels with encapsulated cells were returned to growth media, and gels were allowed to equilibrate for at least 24 h before mechanical testing.

Solid content characterization

Solid content analysis was performed to quantify the final polymer content in each of the hydrogel groups. Both acellular gels were placed in excess PBS and gels with cells were placed in excess growth media for at least 24 h. Equilibrated gel samples were weighed and placed into a desiccation chamber. After at least 48 h, the dried gels were removed and weighed again. The solid fraction is simply the ratio of dry mass to wet mass, and its mathematical inverse is the mass swelling degree, Q.

Toughness analysis

Toughness test for articular cartilage

The cartilage specimens were thawed in the 37°C water bath for 5 min. A crack was made through the bone to the cartilage with a series of custom-designed cutting tool (Fig. 3.3). The thickness of the specimen (B), the width of the articular cartilage (w), the width of the whole specimen (W) and the crack length (a) were then measured with a micrometer under a stereomicroscope (~10X magnification). A new razor blade was used for each cutting to maintain a sharp crack tip. Then each specimen was marked (Fig. 3.4), placed in the Instron (Model 5848, Canton, MA, 50 N load cell), strictly lined up and embedded with fresh PBS solution (Fig. 3.5). A tensile loading was applied with the displacement rate 1.5 mm/min until the specimen pulled apart. The series data of load and displacement were obtained. The ratio a/W was varied from 0.95 to 0.98.

Toughness test for hydrogels

From each type of hydrogel group, eight samples in the same batch were tested. Hydrogel samples were first trimmed to strips by two razor blades bonded together (distance of the two blades were 1 mm). Then each hydrogel strip was glued to a piece of marked closed-cell foam (10 mm in width, 20 mm in length, 1 mm in thickness, see Fig. 3.6) with a cyanoacrylate adhesive (all-purpose super glue from ACE). The crack was made through the closed-cell foam into the gels with the same series of cutting tool (Fig. 3.3). The thickness of the closed-cell foam (B'), the width of the hydrogel (w'), the width of the whole specimen (W') and the crack length (a') were measured with a micrometer under a stereomicroscope (~10X magnification). A new razor blade was used for each cutting to maintain a sharp crack tip. Then each specimen was marked, placed in the Instron (Model 5848, Canton, MA, 50 N load cell), strictly lined up and embedded with fresh PBS solution. The same tensile loading was applied with the displacement rate 1.5 mm/min until the specimen pulled apart. Load and displacement were measured. The ratio a'/W' was varied from 0.95 to 0.98. The effort was made to make hydrogel geometries as similar as possible to the articular cartilage specimen so as to enable apparent fracture toughness comparisons as closely as possible between cartilage and hydrogels.

Calculation for apparent fracture toughness

The models from the American Society for Testing and Materials (ASTM) method #D5045 – 99 (2007) (Standard Test Methods for Plane-Strain Apparent fracture toughness and Strain Energy Release Rate of Plastic Materials) was adopted, elaborated as follows. Apparent fracture toughness was characterized as K_Q in units of MPa·mm½. K_Q was calculated as follows:

$$K_Q = \left(\frac{P_Q}{BW^{\frac{1}{2}}}\right) f(x)$$

where (0.2 < x < 0.8):

$$f(x) = \frac{(2+x)(0.886 + 4.64x - 13.32x^2 + 14.72x^3 - 5.6x^4)}{(1-x)^{\frac{3}{2}}}$$

Where:

P_Q = load determined in ASTM D 5045 – 99 (2007), B = specimen thickness, W = specimen width, a = crack length, x = a /W (Fig. 3.7).

Note that due to the particular geometry of articular cartilage, the range of a/ W would violate the requirement of the ASTM standard. This is recognized as a limitation of the method, but is required because of the physical limitations of the biological material. For this reason, the term “apparent fracture toughness” is used to describe the fracture properties.

Statistical analyses

To compare experimental groups, a single-factor analysis of variance (ANOVA) was performed, followed by a Tukey's Honestly Significant Difference post hoc test when significance was detected. Analysis was performed using the SPSS/PASW 17.0 statistical software package. All quantitative results were expressed as the mean ± standard deviation.

RESULTS

Solid content analysis

The swelling degree for hydrogel groups was shown in Fig. 3.8. Note that each of one type of hydrogels was tested within the same batch. Values are reported as mean ± standard deviation (n = 6). Only the water content in the 6K 20% PEGDA gels was significantly lower compared to other groups in the same molecular weight and cell status (i.e., with or without cells) ($p<0.05$). The water content in the acellular 6K 15% PEGDA gels was significantly 1.96 times as large as that in the acellular 2K 15% PEGDA, 1.14 times large as that in the acellular 6K 15% IPNs ($p<0.005$). The water content in 6K 15% IPNs with 5 million cells/mL was significantly 17% higher than 6K 15% PEGDA gels with 5 million cells/mL ($p<0.005$). The water content in the 6K 20% acellular IPNs was significant 68% higher than that in the 6K 20% acellular PEGDA gels ($p<0.05$). Similarly, the water content in the 6K 15% IPNs with 5 million cells/mL was found to be significantly 17% higher than that in 6K 15% PEGDA gels with 5 million cells/mL ($p<0.05$).

Apparent fracture toughness

The apparent fracture toughness for articular cartilage (n=8) was 348 ± 43 MPa·mm½. The apparent fracture toughness for hydrogel groups (n=8, one group was 7 because one gel strip fell off from the foam before the strip was broken) was relatively lower than that for articular cartilage, varying from 4.04 ± 0.66 to 10.8 ± 1.4 MPa·mm½ (Fig. 3.9). The apparent fracture toughness of articular cartilage was significantly higher compared to the apparent fracture toughness of the hydrogel groups ($p<0.05$).

Note that for each type of hydrogels, they are tested in the same batch. Among the above hydrogel groups, there were four primary observations, which were as follows. First, for the 2K acellular hydrogels, the apparent fracture toughness for 15% PEGDA was considered significantly lower than that of IPN ($p<0.05$). Second, for the 6K acellular hydrogels, the apparent fracture toughness for both 20% PEGDA and IPNs were considered significantly higher than others in the same molecular weight of acellular gels ($p<0.05$). Third, for 15% IPNs, the apparent fracture toughness of 2K cellular gels was significantly lower than other different molecular weight of 15% IPNs ($p<0.05$). Last but not least, the apparent fracture toughness of 6K 20% acellular IPNs was significantly higher than that of 6K 20% acellular PEGDA gels and the apparent fracture toughness of 2K 20% IPNs with 5 million/ml cells was significantly higher than that of 2K 20% PEGDA gels with 5 million/ ml cells ($p<0.05$). However, the apparent fracture toughness of 2K 15% IPNs with 10 million/ ml cells was

significantly lower than that of 2K 15% PEGDA gels with the same cell density ($p<0.05$).

DISCUSSION

The primary objective of this study was to evaluate the apparent fracture toughness for both hydrogels and articular cartilage side by side. To the best of our knowledge, this was the first effort to apply the same method to both articular cartilage and hydrogels with analogous methods for determining apparent fracture toughness. The current study introduced the approach of adhering each hydrogel strip to a piece of thin closed-cell foam to resemble a cartilage specimen, and then test these hydrogels with the modified single edge notch test. The shape of the hydrogel specimen was trimmed in exactly the same way as articular cartilage to avoid difficulties in comparing the apparent fracture toughness value.

With regard to the swelling degree (Q) of the hydrogels, when the molecular weight of hydrogels was increased from 2K to 6K, Q increased. In the study of Johnstone *et al.*, they measured the swelling degree of MW 6K, 12K and 20K PEGDA gels and found out that Q was increasing by increasing molecular weight (56). However, in the current study there was only one exception – Q of 2K 20% acellular PEGDA gels was higher than Q of 6K 20% acellular PEGDA gels, which needed further exploration. Compared to Johnstone's work, the swelling degree of 6K 20% acellular PEGDA gels was only the half of what Johnstone obtained. There was uncertainty of the synthesis of the PEGDA gels. Higher swelling ratios were

beneficial for cartilage matrix production but decreased the mechanical properties of the hydrogels (57).

The major task of this study was to evaluate the apparent fracture toughness for both hydrogels and articular cartilage. In the current study, the apparent fracture toughness for porcine articular cartilage was 348 ± 43 MPa·mm½ using the modified single edge notch test, which was more than 200 times higher than that for bovine articular cartilage measured by the single edge notch test (7). The apparent fracture toughness of adult canine patella cartilage in the study of modified single edge notch test from Chin-Purcell *et al.* was characterized by J integral with the value of 0.14±0.08 KN/m. The current study showed that the apparent fracture toughness of articular cartilage was 31 times higher than that of 6K 20% acellular IPNs, which obtained the highest apparent fracture toughness among the hydrogel groups. Within this perspective, the failure properties of synthesized hydrogels in tissue engineering regeneration clearly need to be enhanced. Thus, factors that can influence the apparent fracture toughness for the hydrogels need to be identified.

The first factor to impact the apparent fracture toughness for hydrogels may be molecular weight. For the same PEGDA concentration, the same cell status (with or without cells) and the same type of hydrogels (PEGDA gels or IPNs), when the molecular weight was increased from 2K to 6K, the apparent fracture toughness increased significantly in most cases. More specifically, it appeared that when the PEGDA concentration was lower (15%) with acellular gels, choosing the higher molecular weight (6K) did not improve the apparent fracture toughness.

PEGDA concentration may be another factor influencing the apparent fracture toughness for hydrogels. For cellular hydrogels, when PEGDA concentration was increased, apparent fracture toughness at the same molecular weight (for both PEGDA and IPN gels) was increased. Especially in 6K acellular hydrogels, the apparent fracture toughness was significantly enhanced when PEG concentration was increased from 15% to 20%. For higher molecular weight of acellular hydrogels, increasing the PEG concentration in IPNs might toughen the hydrogels much better than increasing the PEG concentration in PEGDA gels.

Another factor affecting apparent fracture toughness may be different type of hydrogels (i.e., PEGDA gels vs. IPNs). For acellular hydrogels, the apparent fracture toughness of IPNs was higher than that of PEGDA gels with the same molecular weight and PEG concentration as expected. Because IPNs were two networks (agarose and PEGDA) independent of each other being physically interlocked and combined the mechanical properties for both of the networks, they should show much more resistance to fracture than single network (PEGDA). However, for the cellular hydrogels, most of the IPNs were not tougher than PEGDA gels. One possible reason might be seeding cells as in the following discussion.

The cell density was the most complicated factor affecting apparent fracture toughness for hydrogels. In a few groups, seeding cells increased the apparent fracture toughness while in some other groups seeding cells decreased the apparent fracture toughness. The cell adhesion may tighten the whole structure of hydrogels so as to increase the apparent fracture toughness (58, 59). However, high PEGDA

concentration may interfere with irradiation during photo-polymerization to lower the conversion of PEGDA in a way to decrease the toughness.

There were some limitations on testing both articular cartilage and hydrogels. For testing articular cartilage, the geometry was a limitation in terms of being unable to fit the ASTM standard geometry. However, this limitation is unavoidable, and the method was developed to ensure that the geometry of the specimens was as close to the ASTM standard geometry as possible. In the future, longer term studies will be valuable to evaluate whether extracellular matrix production by cell-seeded gels will significantly improve apparent fracture toughness in this testing regime. Moreover, clearly it is impossible to create apparent fracture toughness tests that will be exactly identical for comparing hydrogels and articular cartilage. Nevertheless, the approach developed here took into consideration a judiciously selected compromise among testing methods available for each material, and the hydrogel testing method was carefully selected to match as closely as possible the modified single edge notch test applied to cartilage.

Overall, a new method was developed to evaluate the failure properties of both articular cartilage and hydrogels in the context of cartilage tissue engineering. Acellular 6K IPNs possessed the maximum toughness among the hydrogel groups examined. However, their apparent fracture toughness was over an order of magnitude less than articular cartilage, so clearly there is much work to do in improving hydrogel fracture properties, unless cells are able to significantly close this gap with the production of functional extracellular matrix in clinically relevant time

periods. Meanwhile factors such as the PEGDA concentration, molecular weight and cell inclusion were found to affect the apparent fracture toughness of hydrogels. Therefore, by providing standard testing methods that accommodate both hydrogels and cartilage, we have a clearer target for the extent by which we must improve the failure properties of hydrogels, which will ultimately lead to better tissue replacements for damaged articular cartilage.

Chapter 4: Evaluation of Apparent Fracture Toughness of 6K 20% Acellular Hydrogels

OBJECTIVE

After testing the apparent fracture toughness of different formulations for the hydrogels, 6K 20% acellular hydrogels brought up the greatest attention. The objective of this chapter was to further confirm the synthesis of the hydrogels and evaluate whether sample source (from different batches or the same batch) would affect the apparent fracture toughness by testing the swelling degree and apparent fracture toughness for 6K 20% acellular hydrogels from different batches (n=5).

MATERIALS AND METHODS

Each of the five batches of 6K 20% PEGDA and IPNs were synthesized and tested using the same procedure of Chapter 3. For the solid content analysis, five batches of 2% agarose gels were also tested to obtain the monomer conversion. Three hydrogel samples from each batch were tested and averaged to obtain the single data point for that particular batch. Then five single data points from the five corresponding batches were averaged to evaluate the swelling degree for a given group. The apparent fracture toughness test was conducted for PEGDA and IPN each from five different batches. No agarose gels were tested in the current procedure for toughness measurement due to the inability to test them. They were too brittle to glue onto the closed-cell foam.

RESULTS

The solid content analysis of the hydrogels was tabulated in Table 4.1 and the swelling degree was reported in Fig 4.1. The five batches of IPNs averaged 90 wt.% of PEGDA in the IPNs, with an average conversion of PEGDA to network polymer in the IPNs was ~54%. The average conversion in PEGDA to PEGDA networks in pure PEGDA gels was ~56% (Table 4.2). The PEGDA concentration was assumed as 20 wt.%, but the final PEGDA content in the PEGDA gel was measured at 10.27 ± 0.48 wt.% (n=5). As significant differences were not observed between mold and final swelled gel dimensions, the PEG content in IPN gels could be roughly calculated by subtracting the solid content of the pure agarose gel from the solid content of the IPN. Agarose gels were composed of ~2.4% agarose, so the PEG content of IPN gels was ~9.64%, which was slightly less than that of pure PEGDA. The swelling degree of PEGDA was significantly 20% higher than that of IPN ($p<0.05$).

The apparent fracture toughness of 6K 20% PEGDA was 7.80 ± 0.93 MPa*mm½ and that of the IPN was 10.8 ± 1.4 MPa*mm½ (Fig 4.2). From the Figure 4.2, it was observed that the reproducibility of the apparent fracture toughness was not good maybe due to the test procedure. The apparent fracture toughness of the PEGDA was significantly 38 % lower than that of the IPN ($p<0.05$).

DISCUSSION

From the results of the swelling degree, about 54% monomer was cross-linked during the synthesis procedure of IPN. However, the crosslinking efficiency was

slightly more than what DeKosky *et al.* obtained (~50%) (5). Variations in oxygen content may have caused a difference in the polymer conversion as oxygen inhibits the free radical polymerization process. The PEGDA source (PEGDAs from Sunbio and Sigma) may have been another reason for the observed difference since the molecular weight distribution may vary from company to company.

From the results of the apparent fracture toughness, the IPN was tougher than the PEGDA as expected. No matter whether the samples were from the same batch (Chapter 3) or from different batches, the apparent fracture toughness of the 6K 20% acellular IPN was significantly ~38% higher than that of PEGDA. Also note that the swelling degree of PEGDA was higher than that of the IPN, which may indicate that lower swelling degree may contribute to higher apparent fracture toughness besides other factors (60, 61).

Compared to the data in Chapter 3, there was no significant difference in the apparent fracture toughness testing for the 6K 20% acellular hydrogels in either the same batch or different batches. No significant difference was observed between 6K 20% acellular IPN from different batches and the same batch. Also compared to Johnstone's work, no significant difference was observed between 6K 20% PEGDA from different batches in this study and those in his study. However, the averaged swelling degree for the 6K 20% acellular PEGDA from different batches was significantly 81% higher than that from the same batch (comparison between the averaged value in Chapter 3 and Chapter 4). That indicate though there was

uncertainty about the synthesis of PEGDA, it would not affect the apparent fracture toughness.

In sum, for 6K 20% acellular hydrogels, the apparent fracture toughness of the IPN, which was composed of only 10 wt.% agarose, in addition to PEGDA, was significantly 20% higher than that of PEGDA. Though there may be more reasons leading to the higher apparent fracture toughness of the IPN, lower swelling degree may contribute to higher apparent fracture toughness. Besides, the reproducibility of the apparent fracture toughness from different batches was confirmed.

CHAPTER 5: DISCUSSION

The crucial objective of this thesis was to evaluate the apparent fracture toughness for both articular cartilage and hydrogels in cartilage tissue engineering. To the best of my knowledge, this was the first effort to apply equivalent toughness measurement methods to both articular cartilage and hydrogels in cartilage tissue engineering. The innovation of this thesis was that apparent fracture toughness method was developed, and factors that play an influence on apparent fracture toughness were identified, following a review of toughness measurement studies on both articular cartilage and hydrogels. Thus, the failure properties of hydrogels must be improved by a significant amount that has now been quantified (over 30 times), which will ultimately lead to better tissue replacements for damaged articular cartilage.

In Chapter 2, different testing methods for apparent fracture toughness were discussed. In a general view of testing articular cartilage, diverse cartilage sources may affect the choice of testing method. For example, in the single edge notch test, articular cartilage from small animals may have limited length for the grips to grab. With the articular cartilage from the ankles of even large animals, the trouser tear test (Mode III) cannot be chosen because the thinness of the cartilage layer and its irregular surface will make it difficult to section into the standard geometry. Another point of consideration is that cartilage is anisotropic, which means different toughness values can be obtained due to the position of the crack opening. However, according to different test mechanisms, the crack opening position may vary.

Of course, the isotropic structure of hydrogels enables us to make any size we want to fit into different testing methods such as the tensile test, tear test, or compression test. Strict attention is necessary to avoid making any micro-cracks when loading those hydrogels into the testing machine, especially when using the trouser tear test or single edge notch test.

To consolidate the two distinct fields of cartilage and hydrogel fracture testing, the best method for both cartilage and hydrogels may be tensile testing based on Mode I because the geometry limitation of cartilage may fail in the trouser tear test and the compression test is unreliable for obtaining reproducible and predictable results. The micro-penetration method works well on articular cartilage with easy manipulation, but it may be limited in testing hydrogels. Though there is no geometry limitation of hydrogels in applying the trouser tear test or compression test, the data may be difficult to reproduce because the hydrogels in cartilage regeneration are normally too soft to make cracks consistently compared to other types such as contact lenses (21, 45-47).

Thus, in Chapter 3, the modified single edge notch test in Mode I was employed, which was based on ASTM standards, to test the apparent fracture toughness for both hydrogels and articular cartilage and thus allowing for more relevant comparisons. The current study showed that the apparent fracture toughness of articular cartilage was 31 times higher than the most promising hydrogels, so clearly there leaves much effort to devote in improving hydrogel fracture properties, unless cells are able to significantly close this gap with the production of functional

extracellular matrix in clinically relevant time periods. In the meantime factors such as the PEGDA concentration, molecular weight and cell inclusion were found to affect both Q and the apparent fracture toughness of hydrogels.

The method was developed in the current study for a specific purpose: to compare apparent fracture toughness directly between articular cartilage and hydrogels. The apparent fracture toughness was not an exact “material property”. It could be evaluated by different methods with different characterizations such as J integral and critical-stress-intensity factor. Therefore, if the same type of hydrogels from the same batch were tested with different toughness methods; different fracture toughness values would be obtained.

However, if several different groups from multiple batches with different formulations (e.g., Group A: 6K 20% acellular PEGDA; Group B: 6K 20% acellular IPNs) were tested with both the current method and other method like single edge notch test, the similar trends should be observed based on the previous preliminary study (data were shown in the Fig A.1). Though in the single edge notch test, fracture toughness of hydrogels could be also measured according to the ASTM standard even in a bigger size, in practical terms the hydrogel samples still either easily slip out of the grips or are crushed by the stress of the grips. Besides, the bigger size requirement of the sample will fail with articular cartilage because of its natural structure limitation. Therefore, no direct comparison could be made between the apparent fracture toughness of articular cartilage and hydrogels if the single edge notch test was chosen.

To confirm the reproducibility from the same batch and different batches, Chapter 4 was set up. It turned out that the apparent fracture toughness could be reproduced either from different batches or the same batch with the comparable results in Chapter 3. The results of swelling degree in Chapter 4 also reflected the reproducibility from different batches to some extent. Though there was uncertainty about the synthesis of PEGDA in Chapter 3, it would not affect the apparent fracture toughness.

In general, the significance of the work was in formulating a methodology to enable the quantification for the apparent fracture toughness value of the synthesized hydrogels and approximate as closely as possible the method used to obtain the apparent fracture toughness of articular cartilage. After reviewing extensive literature on apparent fracture toughness for hydrogels and articular cartilage, a new method was provided to evaluate the failure properties of both articular cartilage and hydrogels in cartilage tissue engineering. Meanwhile PEGDA concentration, molecular weight, and cell existence were found to affect the apparent fracture toughness of hydrogels. 6K 20% acellular IPNs achieved the maximum toughness among the hydrogel groups. However, future work still remains that toughness of hydrogels still needs to be improved compared to the toughness of articular cartilage. Of course combining those factors that affect the apparent fracture toughness of hydrogels will improve the apparent fracture toughness of hydrogels to some extent. Yet the new strategies beyond altering formulations of this particular IPN system will be required, although the inclusion of cells over longer periods of times may close

this gap with the synthesis of neotissue. Thus, future studies would benefit from measuring against *human* cartilage, which may be accessible with IRB approval from other Bioengineering professors at the University of Kansas.

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FIGURES

- CHAPTER 1: No figures.
- CHAPTER 2: Figures 2.1 - 2.8
- CHAPTER 3: Figures 3.1 - 3.9
- CHAPTER 4: Figures 4.1 – 4.2
- CHAPTER 5: No figures

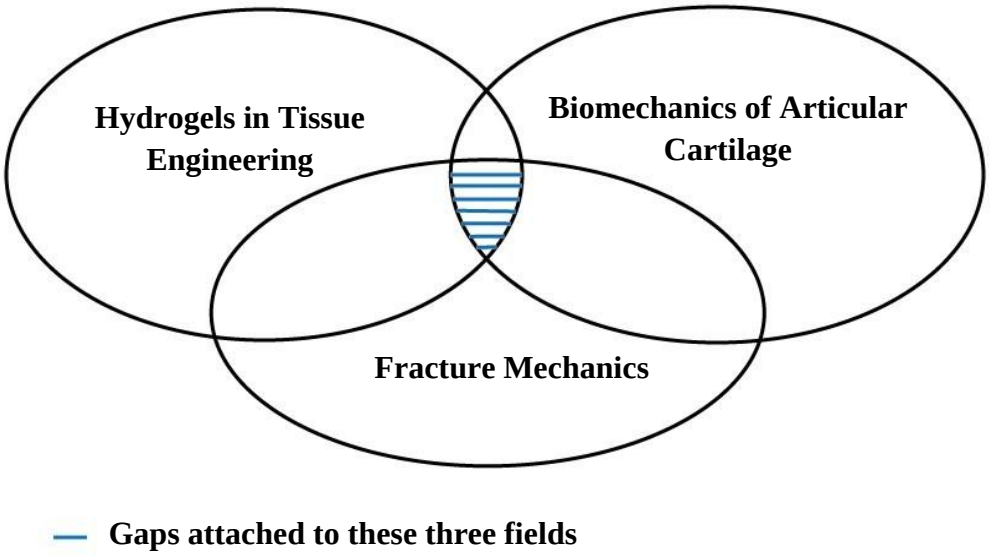


Fig 2.1: Venn diagram emphasizing the distinct fields of 1) hydrogels in tissue engineering, 2) cartilage biomechanics, and 3) fracture mechanics. The purpose of this review is to identify the common ground for these distinct fields, more specifically to understand how to best identify fracture mechanics methods most suitable for evaluating both hydrogels and cartilage. The urgency for identifying this common ground is high in light of the advanced state of hydrogels in cartilage regeneration, where fracture is ready to stand alongside stiffness as a functional design requirement.

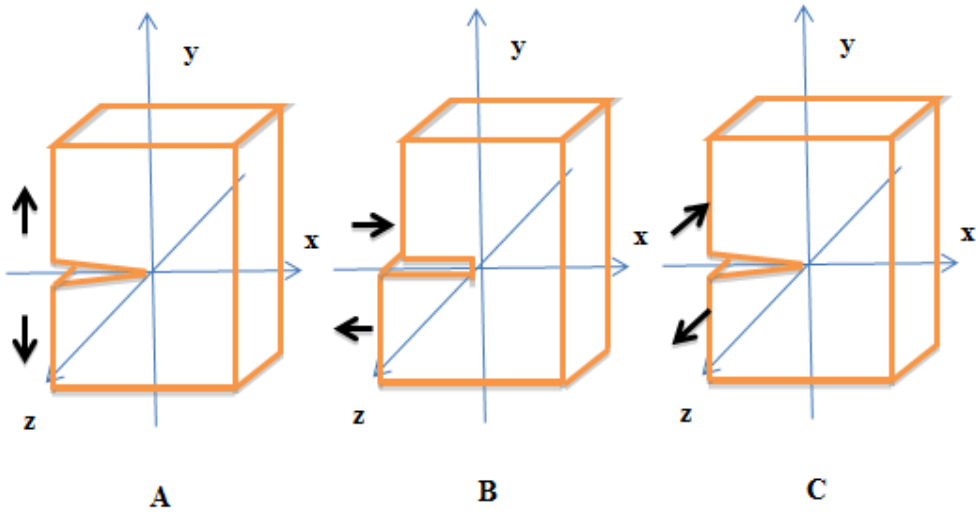


Fig 2.2: Different modes for testing apparent fracture toughness of cartilage summed up by Ahsan (24) and Sah (62) A. Mode I – Opening mode; B. Mode II – Shearing mode; C. Mode III – Tearing mode. Modes I and III have been the preferred methods used for evaluations of cartilage toughness.

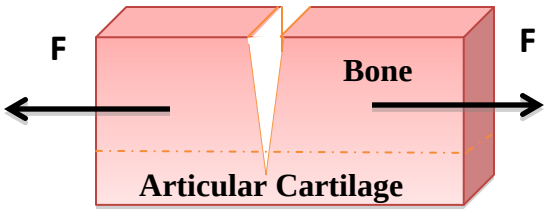


Fig 2.3: Modified single edge notch (MSEN) test for cartilage. Note that the cartilage remains affixed to the bone. The crack made prior to testing extends through the bone and continues a fixed distance into the cartilage, providing a rigid gripping point with the bone.

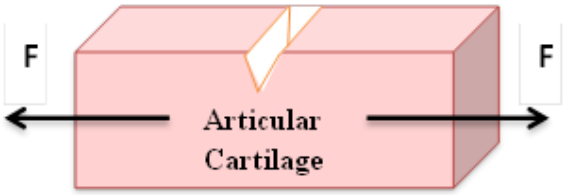


Fig 2.4: Single edge notch test. Note that the cartilage is not affixed to bone unlike the modified single edge notch (MSEN) test. Here, the cartilage is gripped directly.

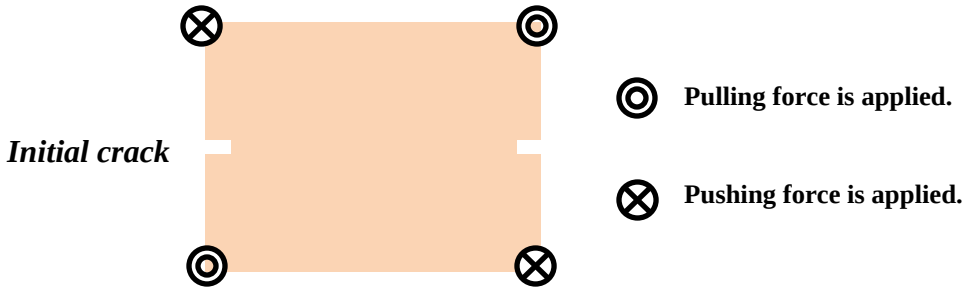


Fig 2.5: Anticlastic plate bending (ACPB) Pull – Apply a force out of the plane; Push – Apply a force into the plane. The ACPB is processed by pulling and pushing the four points in the specimen to propagating the crack, from which trouser tear test is derived.

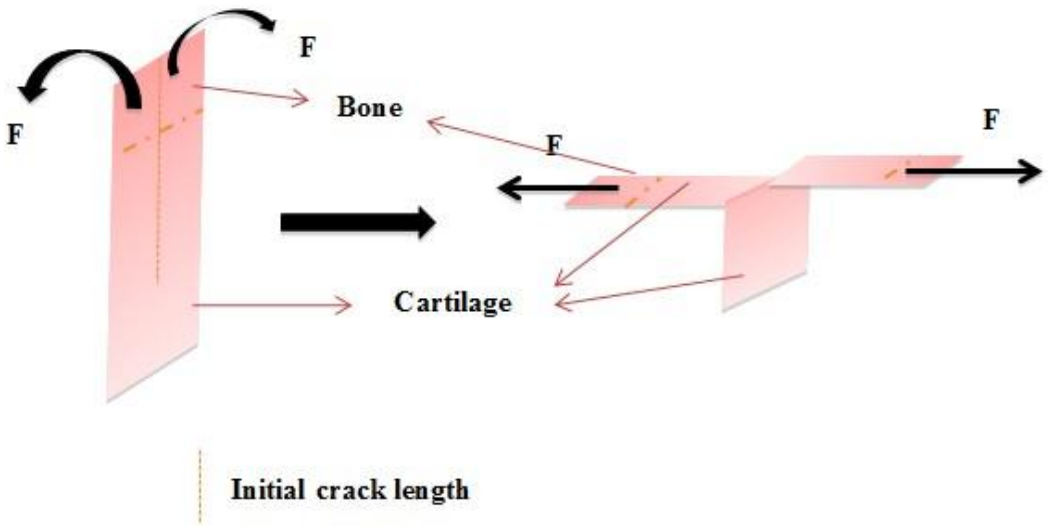


Fig 2.6: Loading mode III – Trouser tear test The grips grab the bone parts to tear through the cartilage.

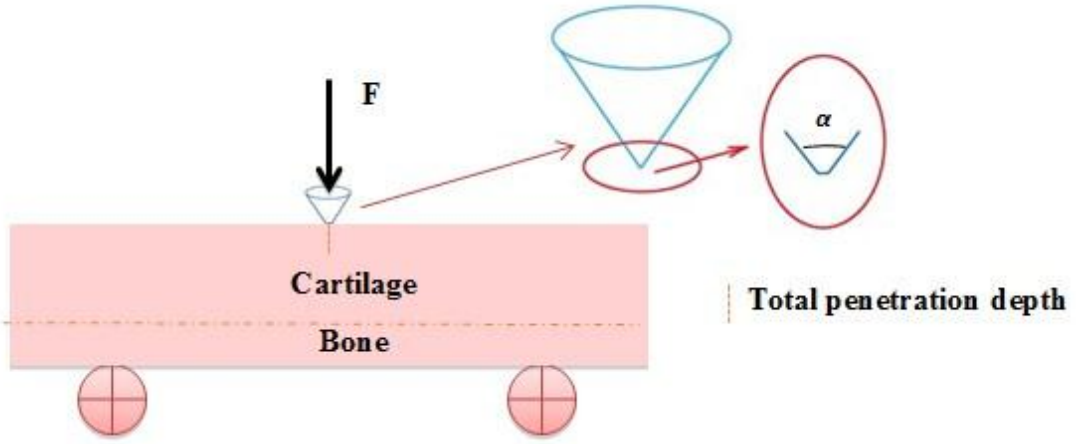
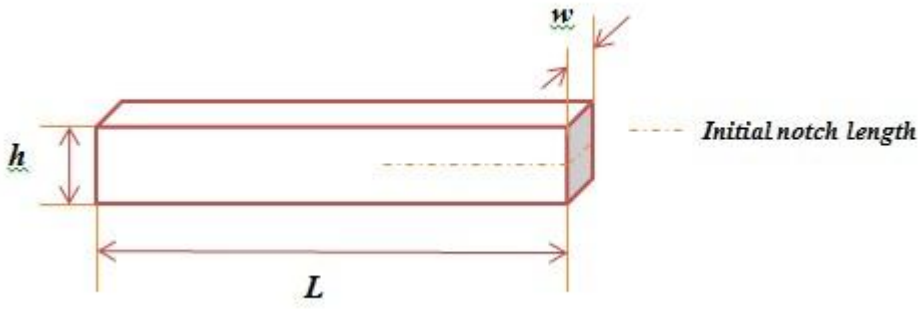


Fig 2.7: Micro-penetration test A penetration or fracture defect in the surface of intact cartilage, which was attached to underlying subchondral bone, was created by a small conical indenter.

A.



B.

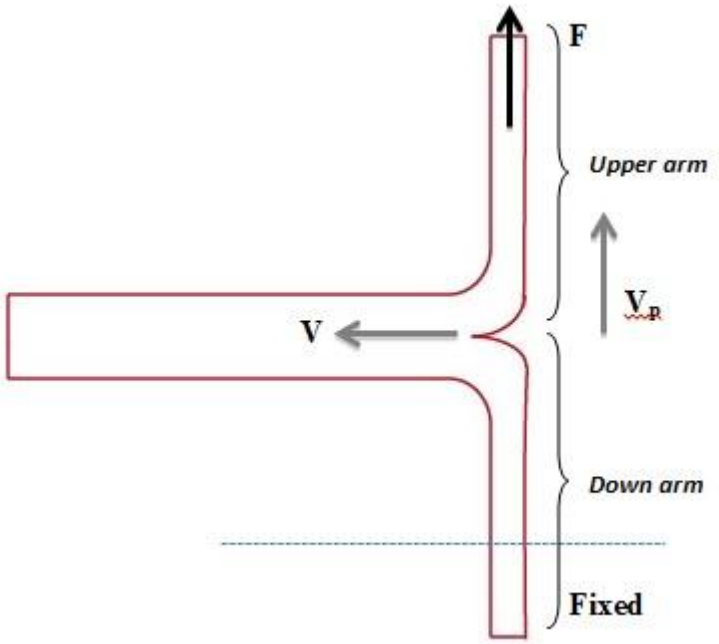


Fig 2.8: Trouser tear test A. Standardized rectangular shape for a trouser tear test with a hydrogel: w = 5 mm, L = 50 mm, h = 7.5 mm, the length of the initial notch is 20 mm; B. Trouser tear test: F is the tearing force, Vp is the pulling velocity and V is the crack velocity.

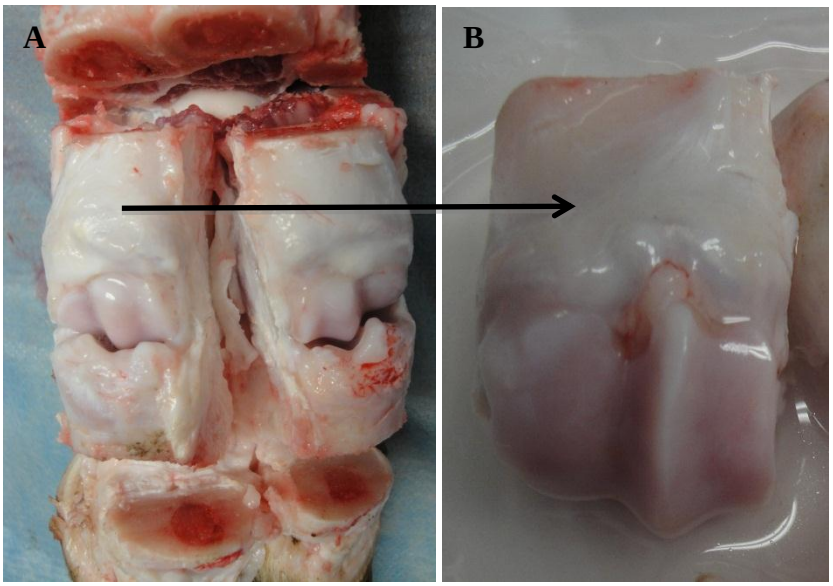


Fig 3.1 Upper joint counterparts were cut off from hog ankles A. Open the hog ankles; B. Upper joint counterpart

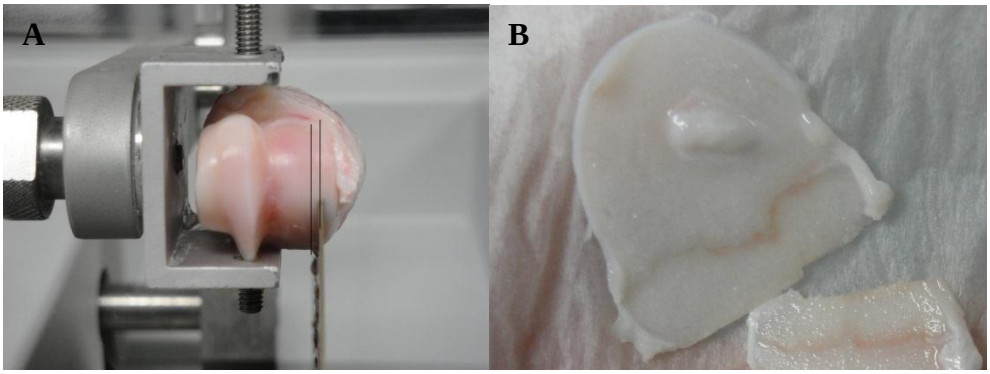


Fig 3.2 Section articular cartilage into pieces A. Section position on each articular cartilage; B. Cartilage pieces

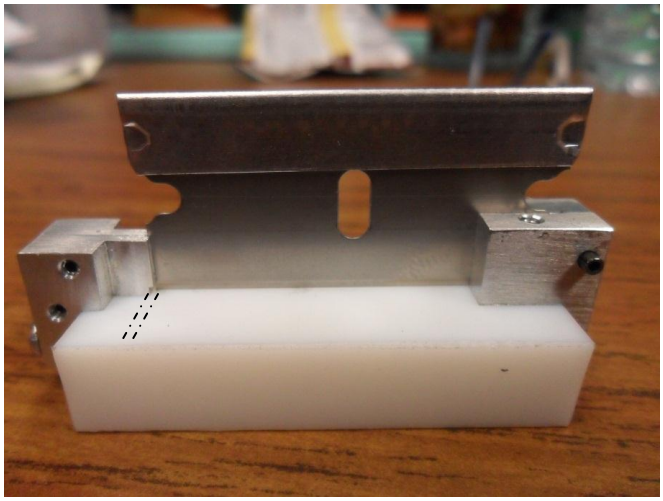


Fig 3.3 Special cutting tool There was a notch (distance was shown between the two black dash lines) before the left head of the blade. There were three cutting tools with three different notch distance known as 0.25 mm 0.36 mm and 0.48 mm. The specimen was inserted on the white plastic plate through the notch. When pressing the blade, the specimen was cut through with part of the specimen remaining inside the notch that was not cut. In such a way, crack lengths were reproducible.

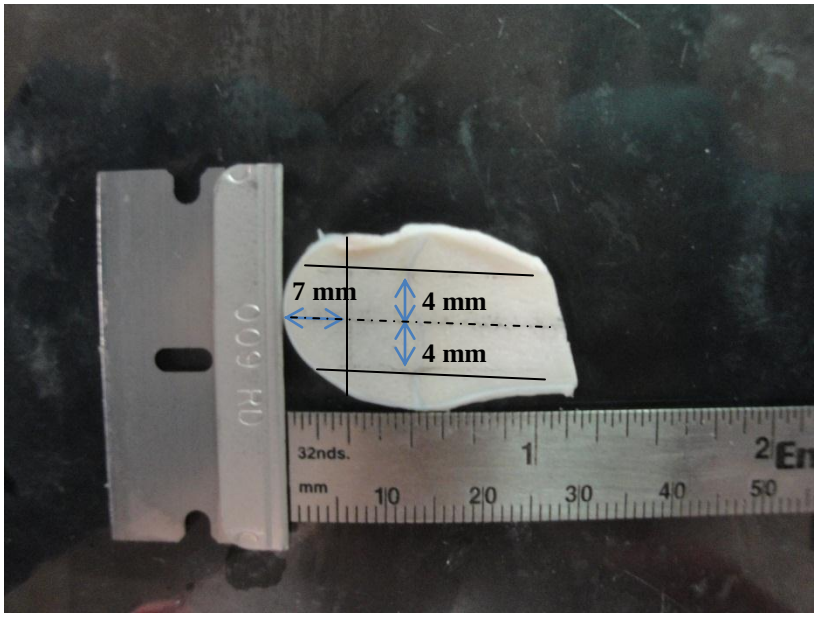


Fig 3.4 Articular cartilage specimen was marked by a pencil. Articular cartilage specimen was marked by pencil so as to line up straight in the grips in the Instron mechanical tester. The crack was opened along the middle of the horizontal dash line. Then grips grabbed the specimen along the vertical black line and the upper and lower horizontal black line respectively (4 mm away the central line and 7 mm away from the cracked cartilage).

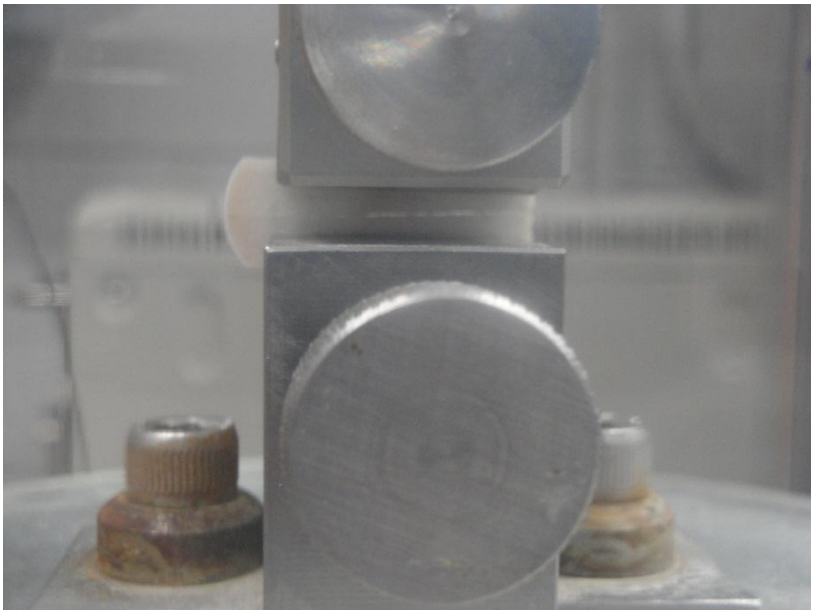


Fig 3.5 Apparent fracture toughness measurement of articular cartilage on Instron embedded with PBS solution. Articular cartilage was placed on Instron along the marks shown in Fig. 3.4. PBS was filled in to equilibrate articular cartilage before testing.

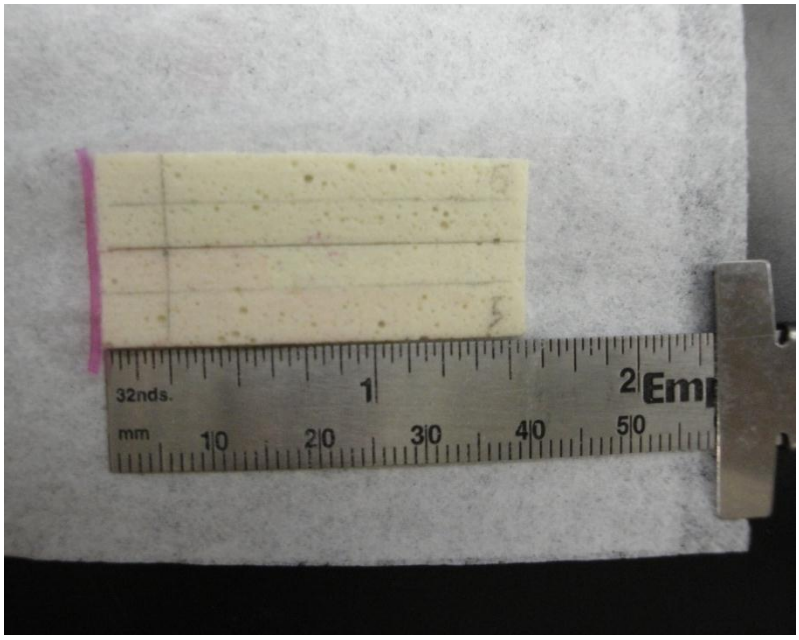


Fig 3.6 Gel specimen Gel strip was glued on the closed-cell foam, which was marked in the same way of marking cartilage specimen.

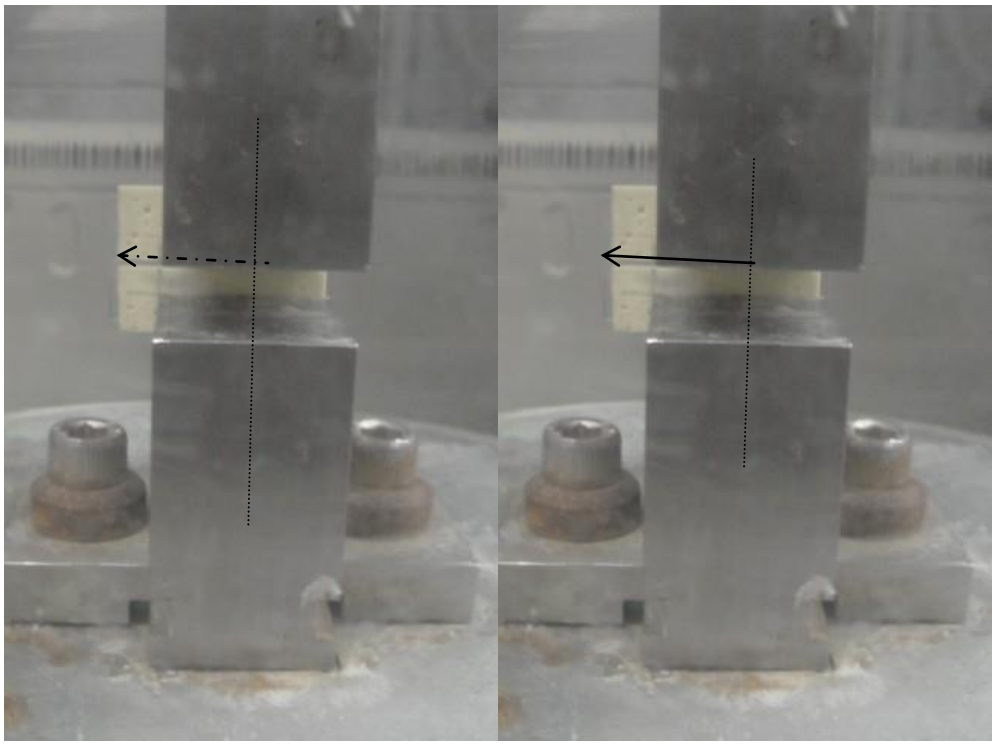


Fig 3.7 The geometry is required in the ASTM standard. a: crack length (dash arrow), which was measured as the horizontal distance from the crack tip to the central line of the grips. W: specimen width (solid arrow), which was measured as the horizontal distance from the end of the specimen to the central line of the grips.

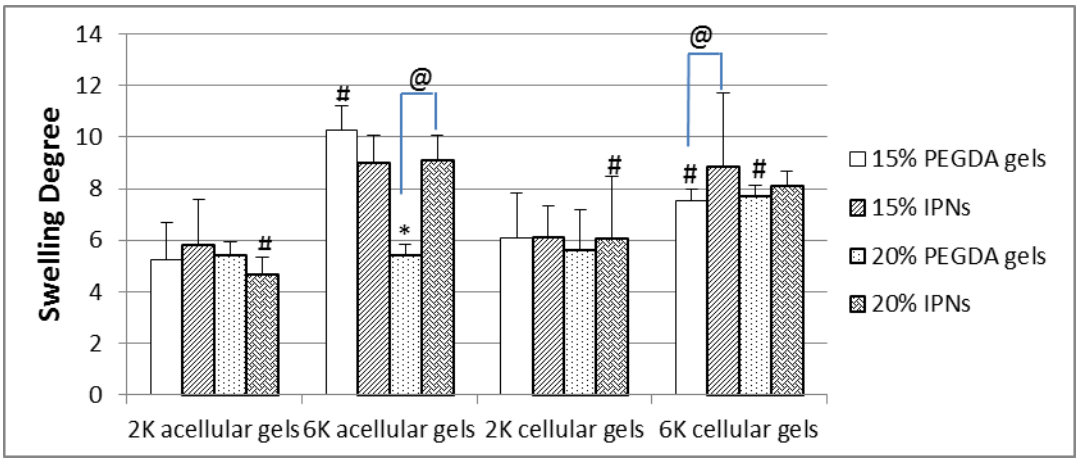


Fig 3.8 Swelling degrees of hydrogels Note that the higher molecular weight acellular hydrogels maintained higher water contents. 2K and 6K refer to the molecular weight of the PEG, and the percentages refer to the PEGDA content. PEGDA = poly(ethylene glycol diacrylate), IPNs = interpenetrating networks. Values are reported as mean ± standard deviation (n = 5 or 6). * = significant difference compared to other groups with the same PEG molecular weight and the same cell status (i.e., with or without) ($p<0.05$). # = significant difference from other groups with the same PEG concentration and the same hydrogel type (i.e., PEGDA or IPN) ($p<0.005$). @ = significant difference between PEGDA gels and IPNs of the same PEG molecular weight, the same PEG concentration and the same cell status ($p<0.05$).

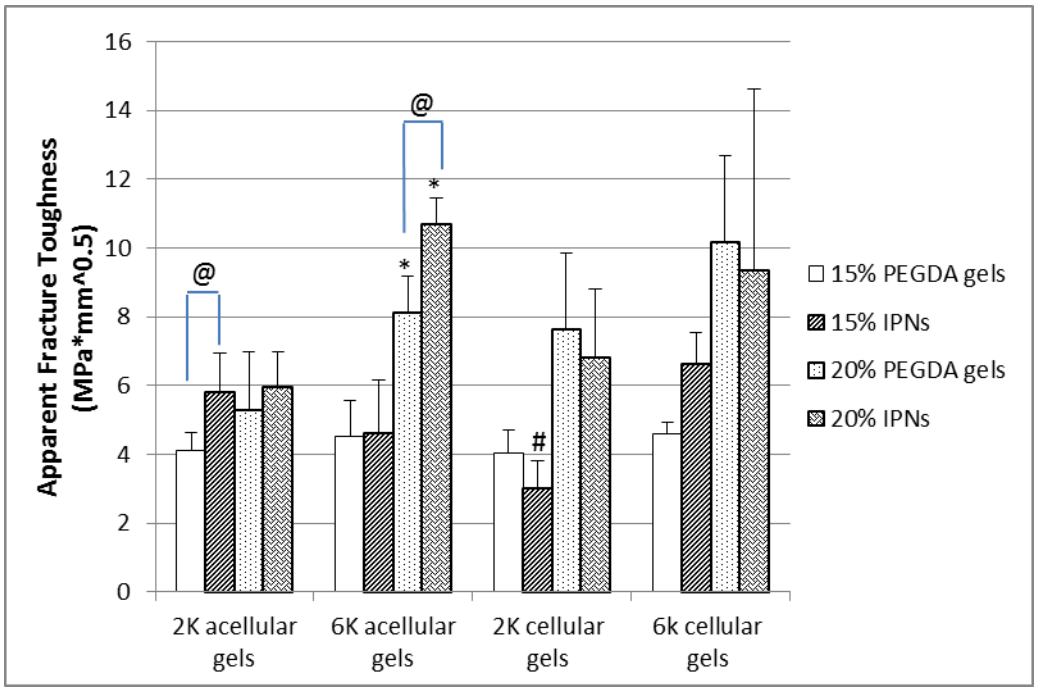


Fig 3.9 Toughness of hydrogels Note that IPNs had a higher toughness than their respective PEGDA hydrogel only in a few select cases. In addition, note that higher toughness values were generally achieved with higher molecular weight and higher PEG concentrations. 2K and 6K refer to the molecular weight of the PEG, and the percentages refer to the PEGDA content. PEGDA = poly(ethylene glycol diacrylate), IPNs = interpenetrating networks. Values are reported as mean ± standard deviation (n = 7 – 8). * = significant difference compared to other groups with the same PEG molecular weight and the same cell status (i.e., with or without) ($p<0.05$). # = significant difference compared other groups with the same PEG concentration and the same hydrogel type (i.e., PEG or IPN) ($p<0.05$). @ = significant difference between PEGDA gels and IPNs of the same PEG molecular weight, the same PEG concentration and the same cell status ($p<0.05$).

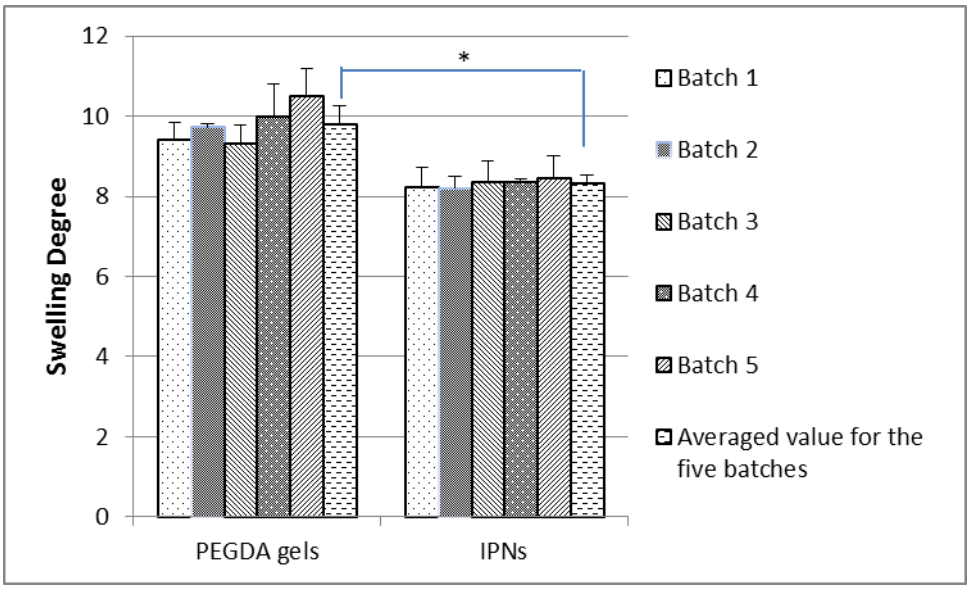


Fig 4.1 Swelling degree of 6K 20% acellular hydrogels. Five independent batches were made for each type of hydrogels. For each batch, three samples were taken to measure the swelling degree. One single point of data for each batch was obtained by averaging the data from those three samples. Then the final data was obtained by averaging the five single data from each batch. Note that 6K refers to the molecular weight of the PEG, and the percentage refers to the PEGDA content. PEGDA = poly(ethylene glycol diacrylate), IPN = interpenetrating network. Final values are reported as mean ± standard deviation (n = 5). There was no significant difference either in the five batches of the PEGDA gels or those of the IPNs ($p<0.05$). * = significant difference ($p<0.05$).

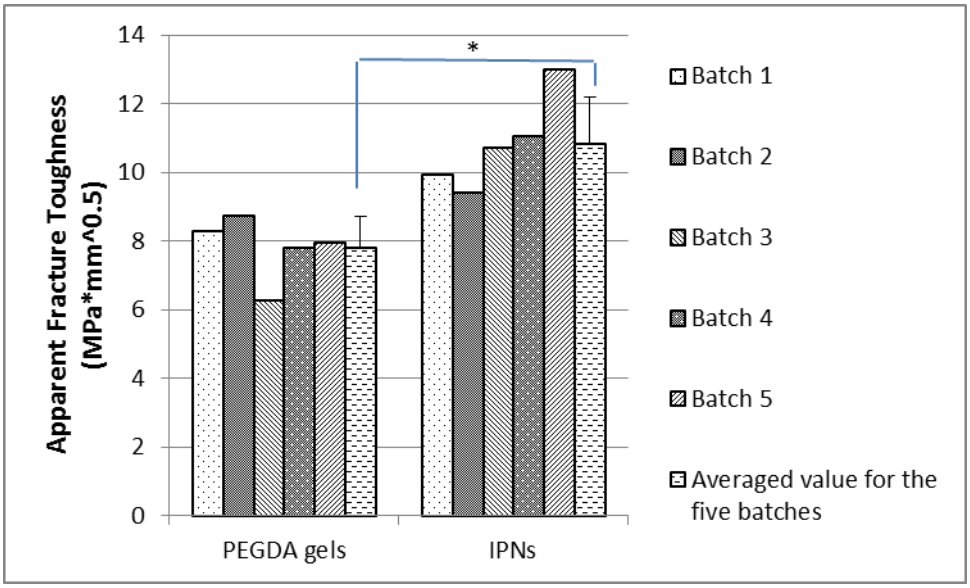


Fig 4.2 Toughness of 6K 20% acellular hydrogels. Five independent batches were made for each type of hydrogels. For each batch, one sample was taken to measure the apparent fracture toughness. The final data was obtained by averaging the five single data from each batch. IPNs had a significantly higher toughness than the PEGDA hydrogel. 6K refers to the molecular weight of the PEG, and the percentage refers to the PEGDA content. PEGDA = poly(ethylene glycol diacrylate), IPN = interpenetrating network. Values are reported as mean ± standard deviation (n=5). * = significant difference ($p<0.05$).

TABLES

- CHAPTER 1: No tables
- CHAPTER 2: Table 2.1 – 2.2
- CHAPTER 3: No tables
- CHAPTER 4: Table 4.1 – 4.2
- CHAPTER 5: No tables

Table 2.1: Comparison of toughness measurements of articular cartilage

Method type	Modified single edge notch test	Single edge notch test	Trouser tear test	Micro-penetration test
Sample resources	Cartilage with bone from the patella of adult mongrel canines*; Bovine articular cartilage with bone from patellae**	Patellae of freshly slain bovine animals	Cartilage with bone from the patella of adult mongrel canines	Bovine articular cartilage from patellae
Geometry of samples	6mm width, 0.2 mm thick in a rectangular shape	7×25mm in width×length, 1-4mm thickness	3mm width, 0.2 mm thick in a rectangular shape	$10\times10\times4mm^3$ including the entire thickness of articular cartilage which was 1-2mm thick
Model	From energy balance, a pseudo-elastic model was establish to measure J integral	The poroe(25)lastic fracture toughness model of articular cartilage was initiated	From energy balance, one dimensional model was establish to measure toughness	Modified standard protocols for Nanoindenter XP
Toughness value	J=0.14-1.2 kN/m Average1070±	Kp_{Ic}=1.83 MPa.mm½	T=J/1.7	Group2: 1102±136 Nm/m^2;

	870 Nm/m^2 *; Average 1030 ± 1019 Nm/m^2 **	(SD=0.8)		Group3: 825 ± 133 Nm/m^2
Advantages	The fracture process can be viewed under microscope allowing to approximate elastic modulus as well	The test process is simple and fast. The fracture process can be viewed under microscope allowing to approximate elastic modulus as well	Calculation is straight forward without complex modeling parameters. Fewer materials are needed.	The toughness obtained is near the surface of articular cartilage. Significantly smaller standard deviation of toughness value is obtained.
Disadvantages	Calculation is not straight forward with complex modeling parameters. More materials will be required.	Calculation is not straight forward with complex modeling parameters. Sample preparation is difficult to some extent	Difficult to view the fracture process under microscope. It simulates unrealistic failure mode.	The tip geometry affects the results.
References	* (30); **(12)	(25); (7)	(30)	(13)

K_{pIc}: Apparent fracture toughness

T: Apparent fracture toughness

J: J integral

Table 2.2: Comparison of toughness measurements of hydrogels

Method type	Single edge notch test	Tensile test	Tear test	Compression test
Sample resources	Alginate gels	MMA-co-45%PEGDMA, 2HEMA-co-2%PEGDMA, MA-co-MMA-co-2%PEGDMA, 100%PEGDMA	PAMPS+PAAM double network gels	Agarose, PEG,IPN
Geometry of the samples	Strips	Dog bone shape	Standard rectangular shape	Circular shape
Model	Energy balance	Integration of the stress vs. strain curve	From energy balance, one dimensional model was establish to measure toughness	Integration of the stress vs. strain curve
Advantages	Different types of fracture energy can be calculated such as work to fracture dissipated outside the process zone	Avoid stress factor caused by the grips due to the geometry of the samples; This method is applied for lots of types of	It is a straight forward testing with standardized geometry of specimen; It is easy to analyze data	It is a straight forward testing with standardized geometry of specimen; It is easy to analyze data

	and the essential work at the process zone	hydrogels		
Disadvantages	It is very likely to crash the gel or lengthening the crack when the gel is loaded	The sample preparation is a time consuming thing; It can only evaluate the total apparent fracture toughness	It is hard to load the gels since it's three dimensional process	The fracture mechanism is different between compression and tension so we cannot compare the value
References	(9)	(8)	(10)	(5)

Table 4.1 Solid content analysis for acellular hydrogels

Sample	Solid content %	Q
2% Agarose	2.412 ±0.083	41.7 ±1.2
6K 20% PEGDA	10.27 ±0.48	9.78 ±0.48
6K 20% IPNs	12.05 ±0.15	8.32 ±0.10

n=5

Table 4.2 PEGDA conversion in acellular hydrogels

Batch No.	Dry Agarose /g	Dry IPNs /g	Dry PEGDAs /g	Wt. PEGDA in IPNs	PEGDA Conversion in IPNs	PEGDA Conversion in PEGDAs
1	0.000237	0.00223	0.00199	0.894	0.554	0.552
2	0.000213	0.00192	0.00200	0.889	0.475	0.555
3	0.000207	0.00242	0.00177	0.915	0.616	0.493
4	0.000233	0.00217	0.00261	0.892	0.538	0.725
5	0.000190	0.00212	0.00170	0.911	0.537	0.472
Average				0.900	0.544	0.559
Std				0.012	0.050	0.099

Appendix 1. Raw data of apparent fracture toughness in single edge notch test

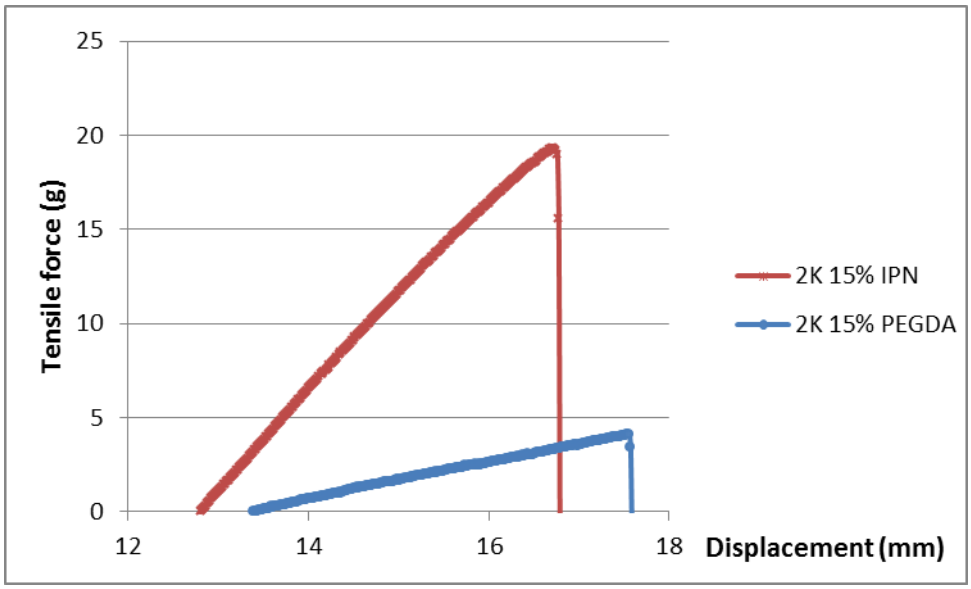


Fig A.1 Raw data of acellular hydrogels. Note that the geometry of IPN and PEGDA were the same as dogbone shape. The crack length over the specimen width was both 0.25.