Exploring Issues for a Cartilage Degradation Model

by

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Abstract

The primary goal of this work was to establish a model system wherein a controlled level of damage is induced to a sample of normal cartilage, such that damage and repair can be observed by the dGEMRIC method of MR imaging. Primarily this included the iterative design of a cartilage tissue holder and testing of its ability to approximate uniaxial diffusion, as well as observation of glycosaminoglycan degradation induced by ADAMTS using dGEMRIC and DMMB assays.

The mean uniaxial degradation rate for 20nM ADAMTS was 0.17 mm/day ± 0.08 (1 δ, n = 4). The shape of the diffusion front was mostly flat, with attributable factors such as vascular structures affecting the shape. These consistencies made ADAMTS an acceptable choice for a degradation agent in the model system.

A final holder design was created that proved largely successful in limiting access of ADAMTS to a single surface, as demonstrated by the flat diffusion/degradation front. This led to a good approximation of uni-axial diffusion and fit well with simple mathematical models of diffusion and previously determined diffusivities of Gadolinium agents in cartilage. A model system using ADAMTS as a degradation agent and dGEMRIC methods of observation can now be implemented for exploration of cartilage's ability to repair itself.

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Chapter 1. Introduction

Arthritis is a common affliction that lowers the quality of living for millions of people. Despite the prevalence of this condition, there is a fundamental lack of understanding of arthritis. A primary reason for this is an absence of knowledge of the pathophysiological processes of cartilage. In particular how chondrocytes, the cells of cartilage, initiate and carry out repair functions to the extracellular matrix.

One method of investigating cartilage repair is the use of enzymes to induce specific damage to regions or molecules of the cartilage matrix. The ability of the cartilage to then repair the induced damage can be monitored. This may include both the rate and the degree of recovery, as well as spatial variations of recovery in regions of the cartilage. In addition, studies could explore whether a 'point of no return' exists for induced damages, and how recovery capacity changes as this induced damage level is approached.

A problem of exploring the inner functions of tissues, in this case cartilage, is the necessity for non-destructive and non-invasive means of examination. Magnetic Resonance Imaging (MRI) provides an excellent means of imaging without physically altering or destroying a sample. Of particular interest here is the dGEMRIC method, a form of microMR imaging that has been developed to study cartilage. Delayed Gadolinium (Gd) Enhanced Magnetic Resonance Imaging of Cartilage (dGEMRIC) uses Gd as a "probe" of the inner function of cartilage. For example, to map the glycosaminoglycan (GAG) content of cartilage, a negatively charged Gd agent can be used. In the case of the dGEMRIC method, the negatively charged gadolinium agent $Gd(DTPA)^{2-}$ saturates a cartilage sample with an inverse distribution to negatively charged GAG. This allows for a determination of GAG distribution using dGEMRIC. The use of dGEMRIC over time would allow for the determination of changes in GAG. GAG chains make up most of the large protoeglycan macromolecules that are part of the cartilage extracellular matrix, and are important for the proper load-bearing function of cartilage. They are lost during disease, and this loss is partially responsible for the loss of functional integrity of cartilage. For this reason, dGEMRIC measurements of GAG concentration may serve as a surrogate measure for the internal function of cartilage.

As noted earlier, one way to gain insight into cartilage repair mechanisms is to examine the recovery of cartilage following enzymatic removal of specific macromolecules. dGEMRIC offers the possibility of visualizing the process during both the degradation and repair phases. The overall objective of this work is to take advantage of dGEMRIC to establish a model system wherein a controlled level of damage is induced to a sample of normal cartilage, such that damage and repair can be observed by dGEMRIC. Accordingly there are two key goals:

- Design and testing of a tissue holder (Chapter 2)
- Establishing the protocol for inducing damage using a representative GAG-cleaving enzyme (Chapter 3)

Chapter 2. Tissue holder design and performance

2.1 Design

Design parameters were set down to provide for a uniaxial diffusion model system. This was achieved primarily by limiting contact of bathing solution to only a single surface of the tissue sample. The other parameters were primarily logistical, and the full list is as follows:

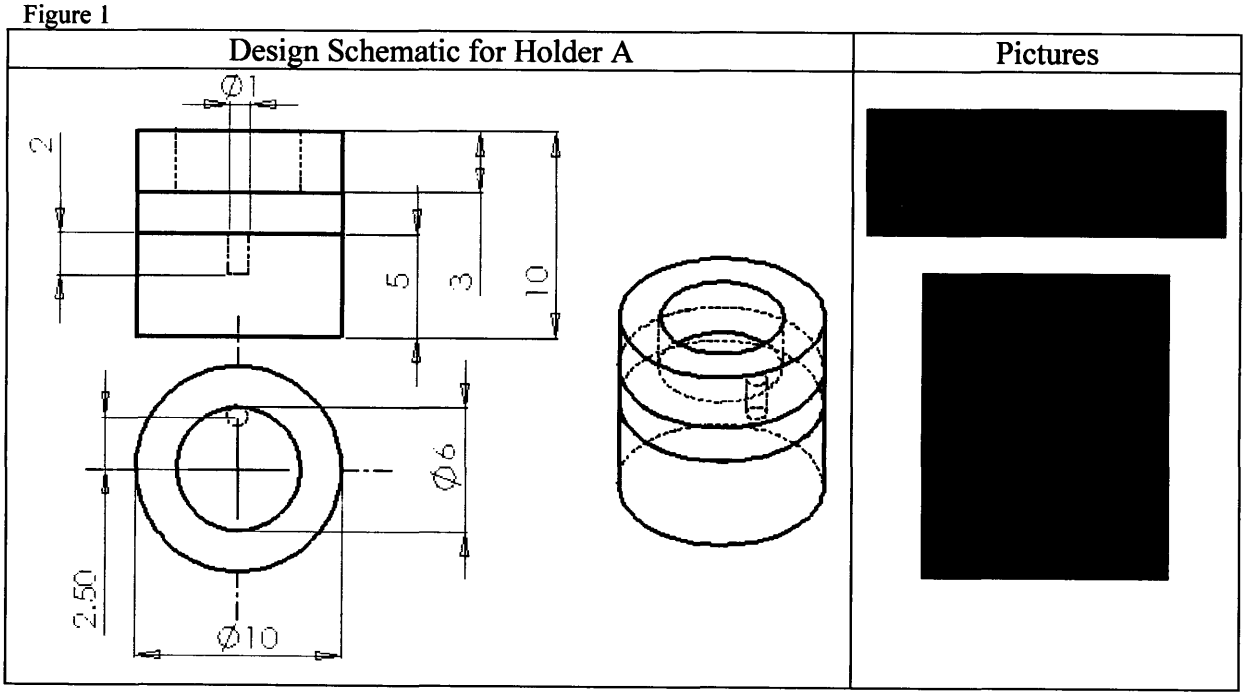
- Diffusion of bathing solution restricted to a single surface
- Organ culture compatible
- MR compatible
- OD less than 9mm (size limitation for highest image quality)
- Fiducial markers to allow for alignment of different scans
- Structurally sound for reuse (autoclaveable, multiple cycles)
- Cost/ease of construction (reliable manufacture of units in timely manner)

As described below, a series of three holders were constructed, making iterative improvements in addressing the design parameters with each new holder.

2.2 Iterative Chamber construction

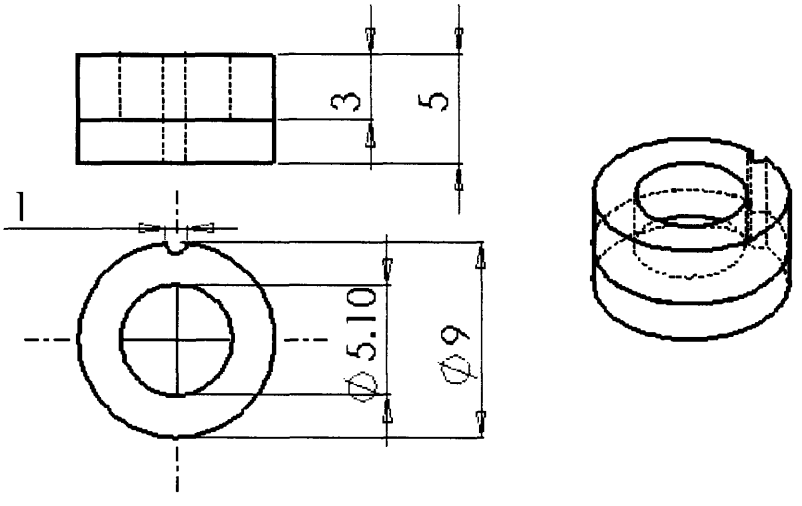
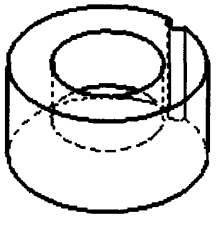
Holder A was designed for a cartilage disk of 6mm in diameter and 3mm in thickness. The OD of the first holder was 10mm exactly, too large to fit into the 9mm ID NMR tube that would need to be placed within the MR coil. Holder A consisted of three pieces: a washer, a disk, and a cylindrical segment, bonded together with 5-minute epoxy. The third segment had a small hole in it that was filled with 1mM Gd in an agar solution. The Gd-active off-center segment allowed for an axial imaging slice to be taken through it, thereby allowing for consecutive coronal scans to be aligned for orientation purposes. The schematic for Holder A is shown in Figure 1. All units are in mm.

Figure 1



Holder B was designed with a small enough OD to fit inside the 9mm ID NMR tube, so a 10mm ID coil can be used, the most sensitive coil likely to be used for future MR studies. This was accomplished by using a 5mm cartilage disk diameter (reduced from 6mm). To accomplish this, a number 7 drill was used to create the washer segment (5.1mm ID), slightly larger than the initial OD of the cartilage to allow for the slight swelling that is normally observed upon equilibration in solution. From preliminary images of holder A, we learned that polysulfone (the material used in all of the holders) was visible in MR images, and could be distinguished from the surrounding solution. Thus, we eliminated the agar-gel-filled fiducial mark, and instead put a groove on the outer perimeter running the vertical length of the holder. In comparison with Holder A, Holder B had the same basic operating principles, but had reduced the number of pieces from 3 to 2, and eliminated the need for agar-filled markers.

Figure 2

Design Schematic for Holder B	Isometric Dwg, Holder C
	

The final evolution involved creating the holder as one entire unit, bypassing the need to epoxy a washer and disc together. This ensured that no solution could leak between the disk-washer interface and maliciously interfere with the uniaxial diffusion model. As shown in Figure 4, test data for Holder B revealed that the epoxy seal was not adequate for multiple uses. Holder C was made from a single piece of polysulfone. The OD of the holder was met by lathing down a larger cylinder of polysulfone (as before). A #7 drill was used to drill 3mm deep along the axis. To solve the bevel problem caused by the shape of the drill head, another #7 drill bit was modified to allow for a 90-degree angle. This allowed holder C to be constructed as a single unit. Compared with Holder B, Holder C improved on the design by eliminating the epoxy, making it easier to construct and making it autoclavable. Although not tested, the life cycle of Holder C is likely the best of the three designs. Holder C is dimensionally identical to holder B and therefore only an isometric diagram of holder C is shown in Figure 2.

2.3 Performance

Key to the performance of the holders is their ability to restrict penetration to the exposed cartilage surface. All the designs achieved this, in principle, by press-fitting the cartilage into a well, so that the only way for solutes to enter the cartilage would be through the exposed surface.

Leaks in the holder, or a loose fit would be expected to degrade the performance. The design strategy was assessed by using MR to monitor the uptake of the uncharged Gadolinium agent Gd(HPDO3A) into the cartilage. An uncharged agent should distribute uniformly throughout the tissue, unlike ionic $Gd(DTPA)^{2-}$.

2.3.1 Methods for Performance Testing

The experimental design is that of a simple diffusion experiment. A small plug of previously frozen bovine nasal cartilage (BNC) was thawed, and a 5mm punch used to create an ≈5mm diameter cylinder of cartilage. This was placed inside of the holder (type B), and the cartilage piece was cut flush to the surface of the holder (5mm OD, 3mm deep). The holder containing the cartilage was put inside an NMR tube, into which 6 ml of Hanks Buffered Saline Solution (HBSS) was added and allowed to equilibrate overnight. After obtaining a baseline image set, the HBSS solution was removed and replaced with 6ml of 1mM Gd(HPDO3A) (Prohance) in HBSS. Images were then obtained repeatedly through the next 24 hours.

All images were obtained using an 8.45 Tesla magnet and a 10 mm ID imaging coil. To measure T1, an "msme_vtr" sequence was used, with 10 relaxation times (100-5000ms)[1]. Total imaging time to collect the data for a single T1 map was about 30 minutes. The image plane was a coronal slice through the shared axis of the cartilage disk and holder. During the uptake period, 48 consecutive T1 weighted sequences were taken over a period of roughly 24 hours. The sample remained in the magnet throughout the 24 hours uptake period. Every effort was made to ensure that the image plane for the uptake images was the same as for the initial baseline image

An in-house image analysis package was used to compute the 49 T1 maps. Briefly, using Matlab 5.3 and the MRI_Mapper program, the data from each set of 10 TRs were fit to a single exponential to compute T1 for each image pixel, and create a T1 map. These maps were computed only for the pixels that were within cartilage (as selected by the user). The resultant T1 maps for cartilage were presented using color scale, overlaid on the image of the entire sample (NMR tube, holder and cartilage). The penetration of Prohance is reflected by the "penetration" of a lower value for T1.

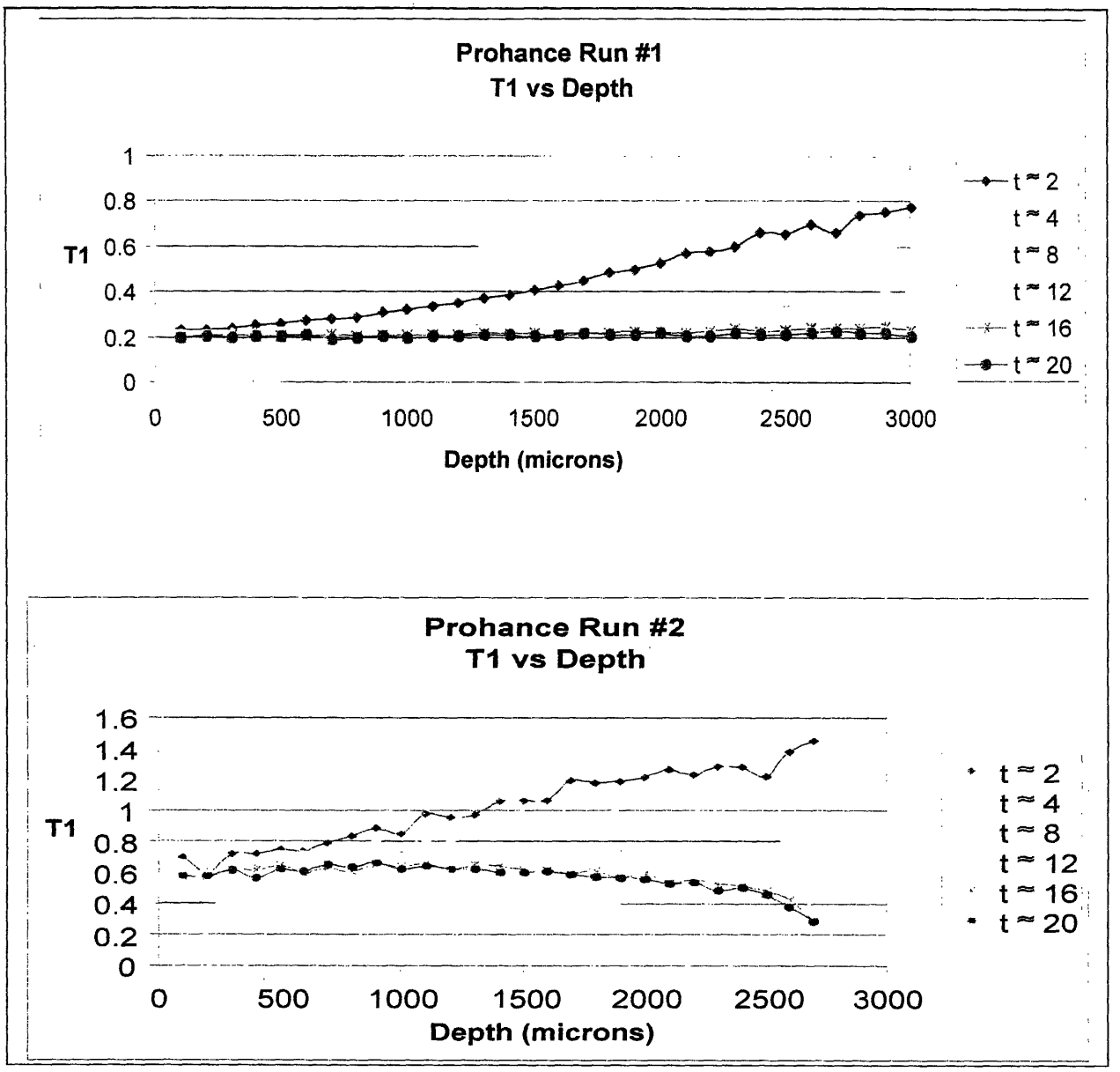
2.3.2 Performance results and discussion

The Prohance uptake study was done twice (Figure 3,4). In both cases the most obvious trend is that T1 increases progressively from the exposed surface towards the bottom of the holder, generally confirming that the chambers functioned as designed. This is because Prohance lowers T1 values, and so higher T1 values at greater depth corresponds to lower penetration of Prohance. (Because of trouble with the scanning software, the baseline image and the first two hours of imaging were lost in the first study, but the trend is nevertheless clear.)

By measuring the T1 values in the bottom-most tissue an estimate of the penetration time was made. For these studies, the time is about 16 hours for 95+% penetration. (Figure 3).

[1] Appendix B, Prohance imaging

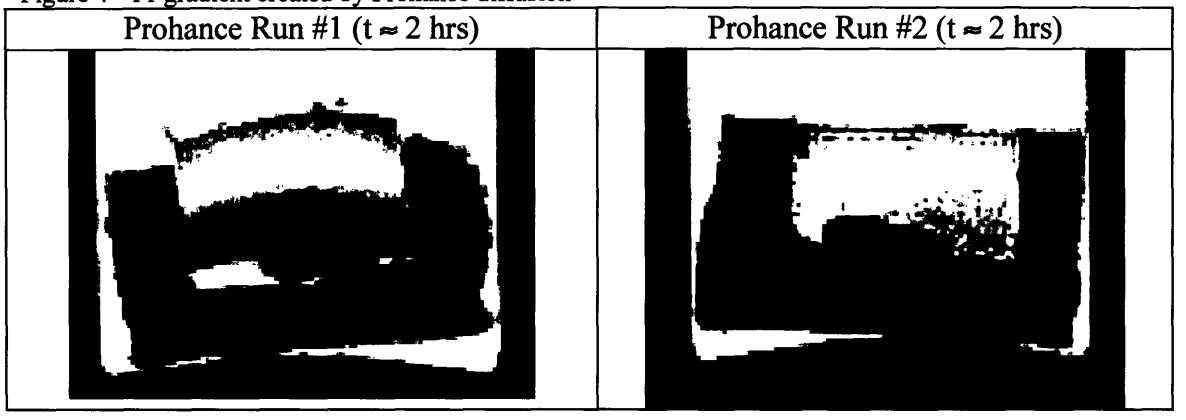
Figure 3 Prohance Runs; T1 vs. Depth



This finding has reasonable correspondence with what would be expected by diffusion, as seen by comparison of the data to theoretical predictions[2]. Assuming a diffusivity of 3E-6 cm^2/s for cartilage, the Prohance diffusion observations are within the range of an idealized diffusion model and values obtained from other researchers (Appendix A).

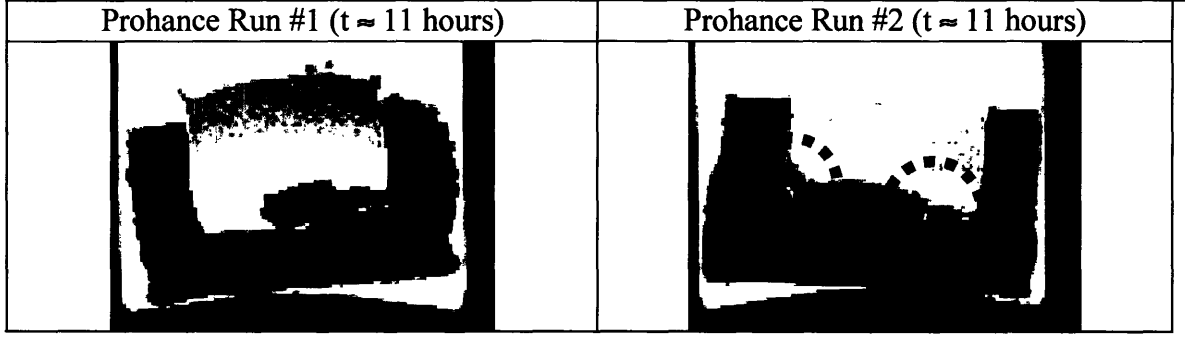
[2] Diffusion model comparison is provided in Appendix A

Figure 4 T1 gradient created by Prohance diffusion



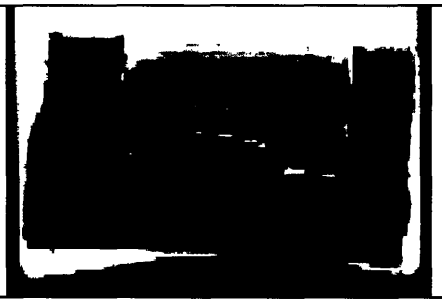
The repeated experiment also served as an assessment of the holder's ability to perform for multiple cycles. Although primarily effective in limiting diffusion, it is apparent from Figures 3 & 5 that the second experiment's bathing solution gains access to the sides and in particular the deepest (2.7mm in the case of Prohance Run #2) circumference ring of the disk, implying a poor washer-to-disk seal. The low T1 values at full depth (circled in pink in Figure 5 and seen in the contrast of the two graphs in Figure 3) of the Prohance Run #2 is unusual even if there was leaking between the washer-disk interface, since it can be assumed that even if a leak exists the top surface of the cartilage is still more exposed to the solution than areas near leaks. When the experiment was complete, the washer was removed from the disk by hand suggesting the original epoxy bond had long since lost any structural significance.

Figure 5 Holder Ability To Limit Diffusion



A possible explanation for the low T1 values in the bottom corners of the Prohance Run #2 (as seen in Figure 5) is the effect of partial-voluming. The coronal slice taken through the sample has a finite thickness. It is possible that for part of that thickness the scan is picking up something else, such as air, and thus averaging the T1 of the obstruction into the value. It also may be that air trapped in the bottom interferes with proper imaging of adjacent areas. If that were the case there may only be a small air pocket trapped beneath the cartilage sample in Prohance Run #2, but it may be responsible for the interference seen as a large black mass occluding the bottom center of the sample.

Figure 6 Prohance Run #2 t ≈1 hr



Note: blue is higher T1 values, yellow/green lower

Both partial-voluming and air interference on adjacent areas may lead to this lowered T1 effect. However, it seems unlikely that either of these is the sole cause. Both of these effects would lead to this phenomenon occurring for all time points, not just later ones. Yet for Prohance Run #2 this effect is not seen in earlier time points. In Figure 6 the bottom left and right corners do not have T1 values lower than any other part of the cartilage.

2.4 Summary of Holder design and performance

In summary, the fabrication of the holders was refined iteratively, with progressive improvements in relation to the design goals (see Table 1).

Penetration of a contrast agent, assessed for Holder B using MR imaging, indicates that the primary site of penetration is the exposed cartilage surface, and the penetration rates are consistent with uniaxial diffusion of the contrast agent. Holder C was never performance tested, as it is identical to holder B with the exception of being a seamless unit. Therefore it was assumed to perform to the same standard as holder B without the possibility of interface leakage.

Table 1 Holder Comparison by Design Parameter

Criteria	Holder:	A	B	C
1	Diffusion of bathing solution restricted to a single surface	NT	1	NT
2	Organ culture compatible	2	2	2
3	MR compatible	2	2	2
4	OD less than 9mm (size limitation for highest image quality)	0	2	2
5	Fiducial markers to allow for referencing image slices from different scans together	2	2	2
6	Structurally sound for reuse (autoclaveable, multiple cycles)	0	0	2
7	Cost/ease of construction (reliable manufacture of units in timely manner) (preferred)	0	1	2

0 = Failed 1 = Criteria Partially Met 2 = Criteria Met NT = not tested

Chapter 3: ADAMTS-induced cartilage degradation

Introduction

The goal is to establish an idealized model system of controlled degradation in a normal cartilage sample. A representative enzyme whose degradation effects are easily monitored is necessary. The proteoglycan-cleaving enzyme ADAMTS was purified by and obtained from collaborators at Pfizer. To evaluate the utility of ADAMTS in establishing a model system, experiments were designed to ask three questions:

1. Does enzyme-induced damage occur in a front-like pattern?
2. What are the temporal and spatial dynamics of induced damage?
3. Is the effect reproducible?

To that end, an initial experiment, ADAMTS #1 was run with a single sample to gather preliminary data, implement the use of a holder design, and as a run through for the imaging protocols. ADAMTS #2 and ADAMTS #3 involved increasing complexity and sample numbers as described below.

Methods

ADAMTS #1 (1 sample, Holder A):

3.1 Degradation Rate, Diffusion Front

	Normal BNC disk
Day 0	• One normal BNC sample was thawed and punched to a 6mm diameter plug. A scalpel was used to cut the sample to a 3mm depth. The sample was then placed in holder type A. • The sample was then equilibrated in 1ml of 1mM $Gd(DTPA)^{2-}$ and Hank's Balanced Salt Solution (HBSS), then placed in an NMR tube. • A baseline image was taken of the sample. The sample was imaged using 10 relaxation times (100-2700ms), msme_vtr (T1 analysis), and saturation recovery[3]. • The sample was kept in an incubator at standard cell culture conditions[4] whenever possible.
Days 1,3,5	• Solution was replaced with 1ml of 1mM $Gd(DTPA)^{2-}$ + 20nM ADAMTS in solution as received from Pfizer.[5]
Days 3,5,7	• Sample was imaged. (All imaging parameters the same for ADAMTS experiments, see Appendix B)
Day 7	• Using Matlab 5.3 and the MRI_Mapper program, the data from each set of 10 scans were fit to a single exponential to compute T1 for each image pixel, and create a T1 map. These maps were computed only for the pixels that were within cartilage (as selected by the user). The resultant T1 maps for cartilage were presented using color scale, overlaid on the image of the entire sample (NMR tube, holder and cartilage). • Degradation rates were determined from the front created by lower T1 values as $Gd(DTPA)^{2-}$ concentration increased while GAG was excised by ADAMTS.

ADAMTS #2 (1 sample, Holder C):

[3] Appendix B, $Gd(DTPA)^{2-}$ imaging parameters
[4] 5% CO_2, 37°C, humidified
[5] 20 ul of 50mM $Gd(DTPA)^{2-}$ was added to 980 ul of 20nM ADAMTS solution as received from Pfizer

3.1.2 GAG loss determination, Degradation Rate, Diffusion Front

	Normal BNC disk
Day 0	• One normal BNC sample was killed by submerging in liquid nitrogen inside a cryo-tube for ≈1 minute, then warming in a 37°C water bath for 15 minutes. This cycle was repeated 3 times. • Punched to a 5mm diameter disk, 3mm deep. • Placed in holder type C. • Sample was equilibrated in 1ml of 1mM $Gd(DTPA)^{2-}$ and HBSS, then placed in an NMR tube. • The sample was kept in an incubator at standard cell conditions whenever possible.
Day 1-5	• Sample was imaged. (Appendix B) • Solution was removed and frozen for later DMMB Assay analysis • Sample placed in 1ml[6] of 10:1 (20nM ADAMTS):(Novex[7] solution) + 1mM $Gd(DTPA)^{2-}$.
Day 6	• Sample was imaged. • Solution was removed and frozen for later DMMB Assay analysis • Cartilage was removed from holder and frozen at –20°C. • Using Matlab 5.3 and the MRI_Mapper program, the data from each set of 10 scans were fit to a single exponential to compute T1 for each image pixel, and create a T1 map. These maps were computed only for the pixels that were within cartilage (as selected by the user). The resultant T1 maps for cartilage were presented using color scale, overlaid on the image of the entire sample (NMR tube, holder and cartilage). Using standard values for cartilage properties[8], GAG concentration was determined for each day using the average T1 value for the cartilage sample as determined from the MR images. Total GAG was computed by multiplying the average GAG concentration by the volume of the cartilage sample (0.06ml). Comparing consecutive days allowed for the calculation of a daily GAG loss (mg). • The Novex solution contains additional salts not present in the solutions with HBSS. All samples that did not have the Novex solution in it had a lower salt concentration, that of normal HBSS. The FCD measurement used to determine GAG concentration from T1 data relies upon the salt concentration of the solution. Using data from ADAMTS #3 and a separate control run, it was determined[9] that the salt concentration of the Novex solution was approximately 240mM. The salt concentration of HBSS is 150mM. *much better • A biochemical DMMB Assay analysis was performed on all aliquots from Days 1-6 to determine daily GAG losses.

[6] Prepared 980 ul of 10:1 (20nM ADAMTS solution):(Novex solution), added 20 ul 50mM $Gd(DTPA)^{2-}$. ADAMTS Lot # KBH0B203

[7] Zymogram Developing Buffer (10X) from Invitrogen, used as directed by Pfizer

[8] See Appendix C Equation 1C

[9] See Appendix C, Novex Salt Concentration

ADAMTS #3 (4 samples, Holder C):

3.1.3 GAG loss determination, Degradation Rate, Diffusion Front

	R A*	R C	NR A	NR C
Day 0	• Four normal BNC sample was killed by submerging in liquid nitrogen inside a cryo-tube for ≈1 minute, then warming in a 37°C water bath for 15 minutes. This cycle was repeated 3 times. • Punched to 5mm diameter disks, 3mm deep. • Placed in holder type C. • All samples equilibrated in 1ml of 1mM $Gd(DTPA)^{2-}$ and HBSS, placed in individual NMR tubes. • All cells kept in incubator at standard cell culture conditions whenever possible. • "R" samples placed on an orbital rocker in incubator.			
Day 1	• All samples were imaged. (Appendix B)			
Day 2-5	• Samples left in incubator.			
Day 6-9	• All samples were imaged. • Solutions removed and frozen for later DMMB Assay analysis			
Day 6-9	-After solution removal placed in 1ml of 10:1 (20nM ADAMTS):(Novex) + 1mM $Gd(DTPA)^{2-}$	-After solution removal placed in 1ml of 1mM $Gd(DTPA)^{2-}$ and HBSS	-After solution removal placed in 1ml of 10:1 (20nM ADAMTS):(Novex) + 1mM $Gd(DTPA)^{2-}$	-After solution removal placed in 1ml of 1mM $Gd(DTPA)^{2-}$ and HBSS
Days 10.5,12	• Same as days 6-9 except 36 hours elapsed between days 9-10.5 and 10.5-12.			

Day 12	• All samples were imaged. • Solutions removed and frozen for later DMMB Assay analysis • Cartilage samples were removed from holder and frozen at –20°C. • Using Matlab 5.3 and the MRI_Mapper program, the data from each set of 10 scans were fit to a single exponential to compute T1 for each image pixel, and create a T1 map. These maps were computed only for the pixels that were within cartilage (as selected by the user). The resultant T1 maps for cartilage were presented using color scale, overlaid on the image of the entire sample (NMR tube, holder and cartilage). Using standard values for cartilage properties, GAG concentration was determined for each day using the average T1 value for the cartilage sample as determined from the MR images. Total GAG was computed by multiplying the average GAG concentration by the volume of the cartilage sample (0.06ml).Comparing consecutive days allowed for the calculation of a daily GAG loss (mg). • The Novex solution contains additional salts not present in the solutions with HBSS. All samples that did not have the Novex solution in it had a lower salt concentration, that of normal HBSS. The FCD measurement used to determine GAG concentration from T1 data relies upon the salt concentration of the solution. Using data from ADAMTS #3 and a separate control run, it was determined that the salt concentration of the Novex solution was approximately 240mM. The salt concentration of HBSS is 150mM. • A biochemical DMMB Assay analysis was performed on all aliquots from Days 6-12 to determine daily GAG losses.

*(R = Rocker NR = No Rocker A = ADAMTS C = Control (no ADAMTS))

3.1.4 Diffusion Front Analysis & Degradation Rate

As described previously, the MATLAB program MRI_MAPPER will generate color-coded maps of GAG concentrations for each designated time for all samples. These maps will be used to analyze the shape of the diffusion/degradation front and to calculate degradation rates. For the front analysis, a later time point image will be taken for each ADAMTS sample. The front will be visible by the abrupt color change signifying the boundary of ADAMTS' penetration and degradation of GAG. The image will be qualitatively assessed to see if the shape of the front is similar to that of later time points (repeatability check). If there is variation within a sample for similar time points, more than one image will be used for that sample. The shape of these fronts will be commented on, with regards to the uniformity of degradation depth and front flatness.

The color GAG concentration maps will be used for all time points on all samples with ADAMTS to determine a degradation rate[10]. It is suggested from previous data[11] that pre-existing vascular structures aids in the diffusion of ADAMTS. With this in mind the degradation rate measures the shortest vertical distance from the surface of the cartilage to the degradation edge. For a given sample, this location will be taken in the same location radially for all time

[10] Appendix D Calculating Degradation Rate

[11] Michelle Farley, presentation on *Model System Development for Cartilage Degradation & Repair*

points. The measured distance will be divided by the number of days since ADAMTS was first added, for a degradation rate value in mm/day.

DMMB Assay data was used as another means to assess GAG losses in samples from ADAMTS #2 and ADAMTS #3. Knowing the GAG losses into solution by the DMMB Assay, it is possible to determine a corresponding idealized degradation rate. Using the following assumptions, theoretical degradation rates were derived for the various ADAMTS runs:

- The 3mm deep 5mm diameter cartilage disk has a volume of 0.06ml.
- BNC has a density of 1.1 g/ml.
- 10% of the mass of BNC is GAG, and this was intact for all of the cartilage before ADAMTS addition.
- Degradation occurred in a front, in uniform depth.
- Degradation was 'all or nothing'. That is degraded cartilage contained 0% GAG, and cartilage not yet degraded contained 10% GAG.

Results

3.2.1 Degradation Rate

The degradation distances for the ADAMTS experiments are in Table 2. ADAMTS #1 having had no Novex solution added, and ADAMTS #2 & ADAMTS #3 (NRA) containing the Novex solution (10:1 ADAMTS:Novex) but not placed on a rocker. ADAMTS #3 (RA) also had (10:1 ADAMTS:Novex solution), and was placed on an orbital rocker while in the incubator. All 'Day' labels refer to the number of days since ADAMTS addition.

Table 2 Degradation Distance From dGEMRIC Images

ADAMTS #1 Degradation Distances (mm)		
Day*	Total	Change Between Measurements
2	0.25	0.25
4	0.50	0.25
6	0.63	0.13

ADAMTS #2 Degradation Distances (mm)		
Day*	Total	Change Between Measurements
1	0.19	0.19
2	0.25	0.06
3	0.38	0.13
4	0.63	0.25
5	0.81	0.19

ADAMTS #3 Degradation Disances (mm)				
Day*	Total		Change Between Measurements	
	RA	NRA	RA	NRA
1	0.38	0.25	0.375	0.25
2	0.50	0.44	0.13	0.19
3	0.75	0.69	0.25	0.25
4.5	1.00	0.88	0.25	0.19
6	1.25	1.00	0.25	0.13

*Days refers to the number of days since the first addition of ADAMTS

DMMB Assay data was also used to determine a degradation rate, as outlined in 3.1.4. Table 3 shows the GAG losses in mg for ADAMTS #2 and #3, and the projected degradation distances corresponding to those GAG losses.

Table 3 Degradation Distances From DMMB Assays

DMMB Cumulative GAG losses (mg)						
ADAMTS #3					ADAMTS #2	
Day	RA	RC	NRA	NRC	Day	
1	0.76	0.17	0.65	0.08	1	0.63
2	1.28	0.23	1.17	0.12	2	1.26
3	1.89	0.29	1.73	0.16	3	1.67
4.5	2.38	0.32	2.20	0.21	4	2.18
6	2.94	0.34	2.69	0.23	5	2.72

Degradation Distance - Estimated From DMMB Assay (mm)						
ADAMTS #3					ADAMTS #2	
Day	RA	RC	NRA	NRC	Day	
1	0.35	0.08	0.30	0.04	1	0.29
2	0.58	0.10	0.53	0.05	2	0.57
3	0.86	0.13	0.79	0.07	3	0.76
4.5	1.08	0.14	1.00	0.09	4	0.99
6	1.34	0.16	1.22	0.11	5	1.24

Degradation Distance - Estimated Change Between Measurements (mm)						
ADAMTS #3					ADAMTS #2	
Day	RA	RC	NRA	NRC	Day	
1	0.35	0.08	0.30	0.04	1	0.29
2	0.24	0.03	0.24	0.02	2	0.29
3	0.28	0.03	0.25	0.02	3	0.19
4.5	0.22	0.01	0.21	0.02	4	0.23
6	0.25	0.01	0.22	0.01	5	0.25

DMMB Assay data was not collected for ADAMTS #1

Using the distances in Tables 3 & 4, degradation rates were determined in mm/day. ADAMTS #1 data was included in the dGEMRIC data but no DMMB Assay data was taken for ADAMTS #1. ADAMTS #1 had the lowest individual degradation rate, and this no doubt contributed to the larger standard deviation seen in dGEMRIC versus the DMMB Assay data.

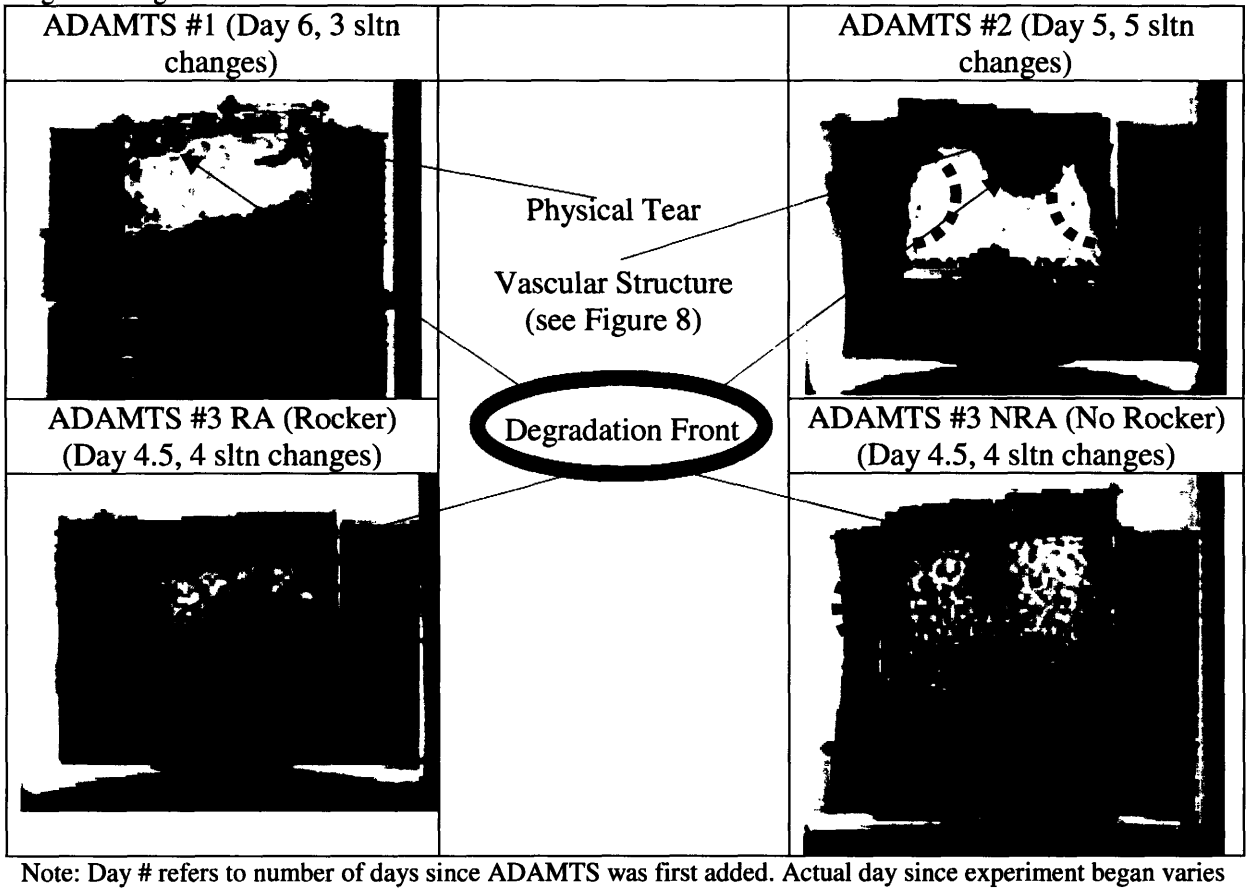
Table 4 Degradation Rate Comparison

	Degradation Rate (mm/day)		
	Mean	δ	Median
dGEMRIC	0.17	0.08	0.17
DMMB Assay	0.23	0.06	0.24

3.2.2 Diffusion Fronts

Figure 7 has GAG concentration maps for various time points from all four of the ADAMTS samples from the three experiments. Three of the four samples present fairly uniform diffusion fronts, with ADAMTS #2 presenting differently.

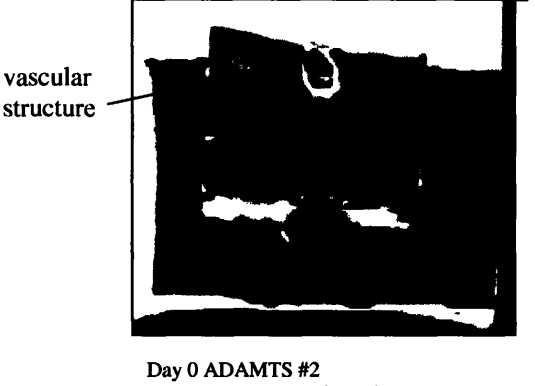
Figure 7 Degradation Fronts



Note: Day # refers to number of days since ADAMTS was first added. Actual day since experiment began varies from experiment to experiment, since they all had different incubation times in HBSS prior to ADAMTS addition.

ADAMTS #2 had an obvious vascular structure in it as seen in the baseline scan in Figure 8. The sample from ADAMTS #1 had a small break in the diffusion front on the far right side, as seen in Figure 7. After the experiment was completed it was scene that the cartilage sample was torn at this location, and it is likely the ADAMTS penetration is due to this. Also, it can be seen that with some of the ADAMTS experiments there is significant degradation along the lateral ends of the sample. In particular in the regions circled with blue in Figure 7.

Figure 8



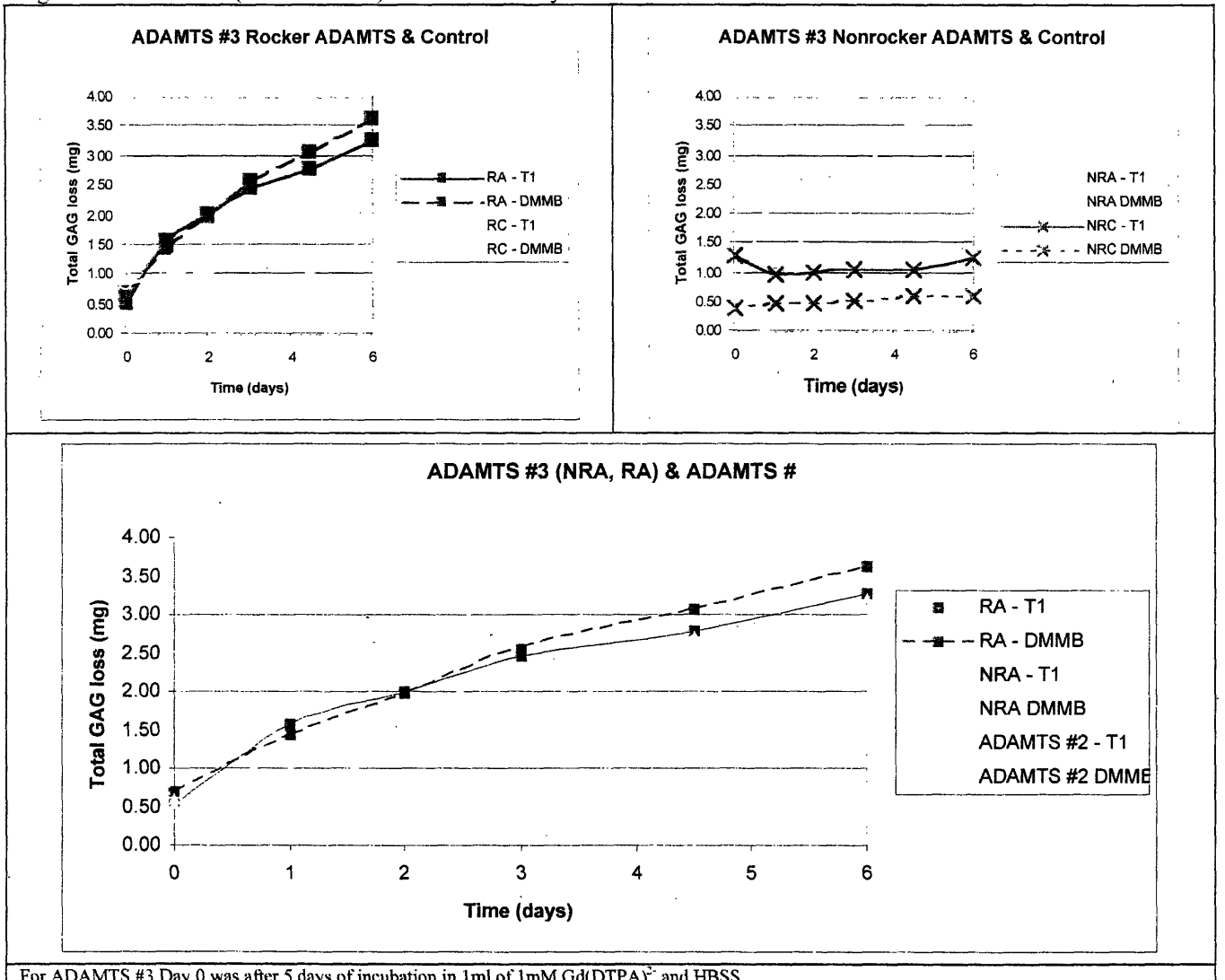
Day 0 ADAMTS #2
(1mM Gd(DTPA)2- in 1ml HBSS)

This is likely indicative of the holder's ability to maintain a seal at the surface, and there is varying success among the samples. (ADAMTS #1 = holder A, all others = holder C) All of the holders used the same technique for placing the cartilage in the well, and so any variation seen is due to individual sample variation.

3.2.3 GAG loss determination, dGEMRIC/DMMB analysis

The GAG losses derived from T1 data generated by the dGEMRIC method is plotted below against DMMB Assay data.

Figure 9 GAG losses (dGEMRIC/T1) vs. DMMB Assay



For ADAMTS #3 Day 0 was after 5 days of incubation in 1ml of 1mM $Gd(DTPA)^{2-}$ and HBSS
For ADAMTS #2 Day 0 was after overnight incubation in 1ml of 1mM $Gd(DTPA)^{2-}$ and HBSS

In ADAMTS #3 all four samples were left in individual 1ml solutions of HBSS and $Gd(DTPA)^{2-}$ for five consecutive days. In Figure 9 the samples in ADAMTS #3 can be seen crossing the x-axis above zero. That is to say on Day 0 the samples had already experienced GAG losses. That is because Day 0 was the day ADAMTS was added, but GAG was lost during the samples 5-day incubation in HBSS and 1mM Magnevist. GAG losses from this were seen

from ranging from 0.39 – 0.84mg of GAG, with the ADAMTS and control sample on the rocker being higher with 0.69mg and 0.84mg of GAG loss, respectively. Non-rocker GAG loss values were 0.53mg and 0.39mg for the ADAMTS and the control experiment, respectively. These values are the GAG lost in a 5-day period without solution change in 1ml of HBSS and 1mM Magnevist. This implies that initially GAG is lost to the solution without the assistance of a degradation enzyme. As seen by the controls in the first two graphs of Figure 9, this leaching effect dies off quickly and little GAG is lost in subsequent days. ADAMTS #2's aliquot for its one-day incubation in 1ml HBSS + 1mM Magnevist was not saved, and so no data is available for it (thus Day 1 is the first GAG loss for ADAMTS #2).

3.3 Discussion

Preliminary data by Michelle Farley had suggested an ADAMTS degradation rate of 0.2mm/day, without using the Novex buffer solution and with no solution changes. ADAMTS #1 (the no-Novex experiment) came in at half of that, at 0.10mm/day. It was anticipated by direction from Pfizer that the addition of the Novex buffer solution would increase the degradation rate. Within the three other ADAMTS experiments conducted here it did so from 0.10mm/day to 0.16-0.17mm/day for ADAMTS #2 & ADAMTS #3 NRA. ADAMTS #3 RA was slightly higher than these two, at an average degradation rate of 0.21mm/day, the same as Michelle's initial experiment without Novex. The majority of samples confirm the preliminary data of a degradation rate ≈0.2 mm/day.

Comparing ADAMTS #3's rocker samples versus non-rocker samples before ADAMTS addition suggests that there is a significant effect on GAG leaching due to the rocker alone. Before the addition of ADAMTS to these samples, they were all incubated in their own solutions of 1ml HBSS + 1mM Magnevist for 5 days without solution changes. During this time, the two rocker samples lost an average of 0.31mg GAG more than the non-rocker samples, according to the DMMB Assay. This implies the rocker motion increases initial GAG leaching into solution.

ADAMTS #3 RA was the only ADAMTS solution on a rocker, and it did have the highest individual degradation rate at 0.21 mm/day. It's not possible with only one ADAMTS sample on the rocker to determine whether the higher GAG loss is due to enhanced ADAMTS transport, or due to enhanced GAG leaching into solution. The difference between the ADAMTS #3 RA sample's degradation rate and the mean was small compared to the standard deviation (difference of 0.04 mm/day, standard deviation of 0.08 mm/day). Given this and the apparent increase in GAG leaching due to rocker use as described in the last paragraph, it is likely there is little increased degradation ability from the rocker's effect of enhancing ADAMTS transport.

The degradation rate as determined by dGEMRIC for the four ADAMTS samples was 0.17 mm/day with a standard deviation of 0.08mm and a median of 0.17mm. This high standard deviation is partially due to ADAMTS #1, which did not use the Novex buffer, and had an individual rate of 0.10 mm/day. Because of the absence of Novex buffer, this solution was expected to have a lower degradation rate. Other variables that were different in the experiments may have contributed to the large standard deviation. These include using different lengths of time before solution changes (2 days between changes in ADAMTS #1, and two 1.5 day periods in ADAMTS #3, vs daily otherwise), and the use of rockers in ADAMTS #3.

The degradation rate as determined by DMMB Assay for the second and third ADAMTS experiments was 0.23 mm/day with a standard deviation of 0.06mm and a median of 0.24mm. This number was expected to be higher than the dGEMRIC degradation rates, because of the assumptions made for the conversion between GAG found in solution (as detected by DMMB

Assays) to a corresponding degraded distance. It is known edges degrade faster due to two surfaces to diffuse from, the holder-washer seal is not perfect, and that vascular structures aid in the diffusion and degradation of ADAMTS through a sample. The idealized assumptions made to determine degradation rates assumed none of these things occurred. Therefore all GAG detected by DMMB Assay in solution was attributed to uniform degradation in a front, even though some of it came from other factors. So the calculation over-estimated the amount of GAG due to uniform degradation, and thus the DMMB Assay determined degradation rate should be higher than a rate measured directly from the tissue being degraded.from the damaged tissue itself (as dGEMRIC does).

With the exception of readily detected indicators, degradation occurred in a relatively flat front, as predicted. The indicators, such as cartilage tears from preparing or vascular structures can be avoided by careful preparation and detection during baseline scanning, respectively. Although not perfect, the data suggests that the ability to reliably degrade the GAG content of the cartilage EC matrix to a uniform depth is feasible.

A limitation of GAG losses determined through the dGEMRIC method is the reliance on the T1 matrix generated from the MR imaging. As seen in Table 3, interference can obstruct or partially obstruct the full view of a cartilage sample. This may significantly impact the T1 average for the slice, especially in later stages of degradation where the occluded regions of cartilage (at the bottom of the sample, where air is trapped in between the holder and the sample) may have very different T1 values than the degraded upper regions of cartilage. This is primarily a problem when the obstruction changes in size and shape from day to day, impacting the T1 cartilage averages differently everyday. ADAMTS #3 NRA images had this obstruction problem most often. This may explain why in Figure 9, the ADAMTS #3 NRA seems to demonstrate a 'wavy' slope of large jumps in degradation and then very small increases. Day to day air movement would be blocking out different regions of the cartilage sample, this radically changing day to day GAG losses. If the wavy nature of the slope is due to this, it is likely that the overall trend is still valid because the summation of variations would cancel eachother out.

The air bubble effect was a twofold problem. By raising the cartilage from the bottom of the well, it allowed solution to enter and prematurely expose cartilage to the degradation agent. In addition it lowered the stability of the cartilage in the holder, possibly promoting more circumferential diffusion of the bathing solution. As noted it presents a problem with GAG loss determination via imaging, but it is unlikely to have effected degradation depth measurements. That is because it was apparent that greater penetration occured at the top corners of the cartilage, due to two surfaces allowing diffusion for ADAMTS. That is why the shortest vertical distance was used to determine degradation rate as described in 3.1.4 and Appendix D.

Attempts were made to remedy the trapped air problem by placing the cartilage into the holder while submerged in solution. Alternatively, a syringe needle was placed in between the cartilage and well wall to evacuate air while the cartilage was being placed into the holder. Neither of these methods worked completely, but samples fit while submerged appeared to have the smallest presence of air bubbles (Table 3, ADAMTS #2).

Chapter 4: Thesis summary

The primary goals of this work were to design and test a tissue holder, and to establish a protocol for inducing damage using a representative GAG-cleaving enzyme. This is to support the later goal of using dGEMRIC to monitor the repair of the EC matrix of cartilage under

different states of degradation. The first goal of design and testing of a tissue holder led to an effective holder after several iterations, and it allowed for a good approximation of a uniaxial diffusion model in the degradation experiments.

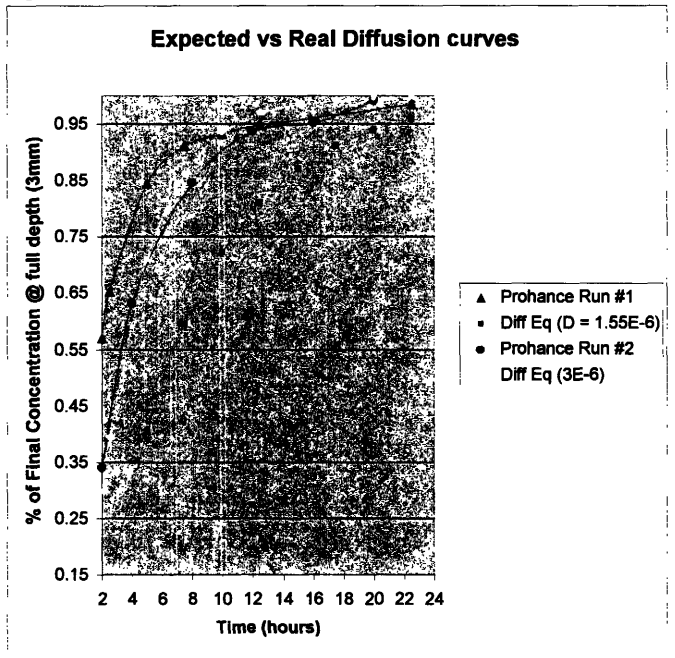
The degradation rates for 20nM ADAMTS was a mean value of 0.17 mm/day ± 0.08 (1 _, n = 4). DMMB Assay GAG losses and a theoretical degradation model yielded slightly higher results, at 0.23 mm/day ± 0.06 (1 _, n = 3). The shape of the diffusion front was mostly flat. This data suggests that ADAMTS is a good enzyme to induce controlled damage to a specific depth of a cartilage sample.

Degradation rates and GAG losses that are empirically and rationally consistent with eachother shows that ADAMTS' use can be largely controlled. Careful sample preparation and baseline scanning for significant vascular structures can select for samples to best approximate an ideal diffusion front, allowing for a degradation penetration that is uniform in depth. A robust protocol can now be established for further testing of the degradation enzyme ADAMTS, or other advances of the model system.

Appendix A: Diffusion Model

In Figure 7 both Prohance runs are plotted as a percentage of diffusion completion versus time. Using an idealized diffusion equation as outlined below, a previously derived value for the diffusivity of Prohance in cartilage was plotted. (D = 1.55E-6 cm^2/sec, seen in pink below)[12] Using this diffusion equation a 'best fit' value was qualitatively determined to be 3E-6 cm^2/sec, which is within the range of the value determined by Gillis et al. The two Prohance runs are seen in blue.

Figure 10



[12] *Relaxivity and Diffusion of Gadolinium Agents in Cartilage*, Gillis, Gray, Burstein.

Diffusion in a semi-infinite medium[13] with boundary conditions:

- Interface kept at constant concentration C_0
- Initial concentration is zero throughout the depth of the cartilage (medium)
- $0 < x < l$ is the distance covering the medium (l is the depth of the cartilage, 3mm in this case)
- Distances l and greater are the infinite solution
- There is no diffusion across the plane x = 0 (bottom of the 3mm cartilage depth)
- Concentration (C) is a function of depth (x)

$$\overline{C} = C_0 * \sum_{n=0}^{\infty} (-1)^n erfc \frac{(2n+1)l - x}{2\sqrt{(Dt)}} + C_0 * \sum_{n=0}^{\infty} (-1)^n erfc \frac{(2n+1)l + x}{2\sqrt{(Dt)}}$$

Numerical summation was performed using Microsoft Excel for n = [0-7]. Values at n = 7 converged to near zero for all times, with the largest value less than 1E-6 of a percent of the final summation.

Appendix B: MRI Parameters

Gd(HPDO3A) (trade name Prohance, from Bracco Diagnostics) Runs:
Used to test design parameters of holder
Bruker 8.45 Tesla Magnet
10mm coil
Saturation Recovery
Sequence – msme_vtr
TE: 15ms
TR (#=10): (100, 150, 300, 400, 600, 900, 1400, 2000, 3200, 5000)
Averages: 1
Slice Package = 1 * 0.5mm coronal
Matrix = 128 x 128
FOV = 1.28 cm

$Gd(DTPA)^{2-}$ (trade name Magnevist, from Berlex Imaging) Runs:
Necessary for dGEMRIC imaging of GAG losses due to degradation
Bruker 8.45 Tesla Magnet
10mm coil
Saturation Recovery
Sequence – msme_vtr
TE: 15ms
TR (#=10): (100, 125, 175, 275, 375, 475, 600, 900, 1800, 2700)
Averages: 2
Slice Package = 1 * 0.5mm coronal
Matrix = 128 x 128
FOV = 1.28 cm

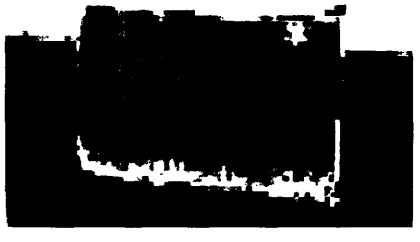
[13] Crank, The Mathematics of Diffusion 2nd Edition, Equation (2.54) (pg 22)

Appendix C Novex Salt Concentration

A control scan was run to determine the effects of the Novex solution's salt concentration on the computation for the GAG concentration. The Fixed Charge Density (FCD) is a set cartilage property that is needed to make the calculation for GAG concentration from T1 data. The FCD determination calculation from T1 data is affected by the salt concentration, and the Novex buffer contains a greater (and unknown) salt concentration than HBSS. Therefore a baseline scan was taken of a cartilage sample that was prepared in the same manner as used for all samples in ADAMTS #3. This sample was then equilibrated in 1ml of HBSS and 1mM Magnevist overnight. The following day the sample was scanned following the Magnevist parameters used for all ADAMTS experiments. After the scan the solution was replaced with 1ml of 10:1 (HBSS):(Novex) and 1mM Magnevist. Previously for ADAMTS experiments the solution for ADAMTS samples were made up of (20nM ADAMTS):(Novex) and 1mM Magnevist. The control was left overnight in its HBSS/Novex/Magnevist solution and scanned the following day. The resulting images are below.

Figure 11 Novex Control Run

Day 0: Baseline Scan (1 Day in HBSS + Magnevist)



Day 1: Novex Addition (1 Day in 10:1 HBSS: Novex + Magnevist)

It can be seen that the day after incubating in the HBSS/Novex/Magnevist solution the sample has unusually low T1 values, normally indicative of lowered GAG values. Although the T1 values are lower, the GAG concentration is not[14]. Normally, a T1 average is taken of the whole slice and converted to a GAG concentration. Equation 1C below represents the general format to calculate GAG concentration. In the baseline scan the solution had a T1 value of 0.181, T1 cartilage average of 0.492, salt concentration of 150mM (standard for all solutions without Novex) and a final GAG concentration of 110mg/ml. The post-Novex scan had a solution T1 value of 0.190, T1 cartilage average of 0.362, an unknown salt concentration, and an assumed GAG concentration of 110mg/ml (unchanged from the day before). With this data, it was determined that the Novex solution had a salt concentration of 240mM.

Equation 1C

$$[Gd](bath) = 1/R(bath)*(1/BathT1^{+} - 1/SalineT1)$$

[14] Based on control data from ADAMTS#3, the assumption is made here that no GAG losses occur between Day 0 and Day 1 scans

[Gd](cartilage) = 1/R(cartilage) * (1/T1Gd$^-$ - 1/T1Gd$^+$)
FCD = 2 * [salt concentration] * ([Gd]bath – [Gd](cartilage))/([Gd]bath * [Gd](cartilage))
[GAG] = -0.5 * .5025 * FCD

T1 Saline = 2.75; T1 Cartilage (equilibrated in saline) = 1.6;
Relaxivity Cartilage = 4.6; Relaxivity Bath = 4.35
All other T1 values are measured using MR Imaging

Appendix D Calculating Degradation Distances

The degradation rates for the ADAMTS samples were calculated as follows:

- The image file was imported into Adobe Photoshop.
- A reference line was drawn that runs the entire depth of the holder. This reference line corresponds with 3mm. All of the holders were manufactured with a well depth of 3mm (in the case below, the longer of the two lines).
- The reference line was then subdivided into 48 even parts, digitally. Each of these subdivisions corresponded to a length of 1/16th of a mm.
- Another line was drawn running from the cartilage's top surface down to the edge of the degradation front (the shorter of the two lines below). The shortest distance was taken, therefore areas like those circled were avoided. This was done because most jumps in the degradation front were due to vascular structures and edge effects, not a product of uniaxial degradation through the EC matrix.
- The line measuring the depth of the degradation front penetration was then compared to the reference line. A degradation penetration depth was then measured. This was then the cumulative degradation distance for that sample on that day. The difference of consecutive days measurements was the daily distance degraded. The measurement error was 1/16th of a mm, the smallest subdivision of the reference line.
- This was done for all time points for all ADAMTS samples. The mean value, standard deviation, and median value were calculated (Table 4).

Figure 12