Depth-Dependent Glycosaminoglycan Concentration in Articular Cartilage by Quantitative Contrast-Enhanced Micro–Computed Tomography

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Abstract

Objective. A quantitative contrast-enhanced micro–computed tomography (qCECT) method was developed to investigate the depth dependency and heterogeneity of the glycosaminoglycan (GAG) concentration of ex vivo cartilage equilibrated with an anionic radiographic contrast agent, Hexabrix. Design. Full-thickness fresh native (n = 19 in 3 subgroups) and trypsin-degraded (n = 6) articular cartilage blocks were imaged using micro–computed tomography (μCT) at high resolution (13.4 μm³) before and after equilibration with various Hexabrix bathing concentrations. The GAG concentration was calculated depth-dependently based on Gibbs-Donnan equilibrium theory. Analysis of variance with Tukey’s post hoc was used to test for statistical significance (P < 0.05) for effect of Hexabrix bathing concentration, and for differences in bulk and zonal GAG concentrations individually and compared between native and trypsin-degraded cartilage. Results. The bulk GAG concentration was calculated to be 74.44 ± 6.09 and 11.99 ± 4.24 mg/mL for native and degraded cartilage, respectively. A statistical difference was demonstrated for bulk and zonal GAG between native and degraded cartilage (P < 0.032). A statistical difference was not demonstrated for bulk GAG when comparing Hexabrix bathing concentrations (P > 0.3214) for neither native nor degraded cartilage. Depth-dependent GAG analysis of native cartilage revealed a statistical difference only in the radial zone between 30% and 50% Hexabrix bathing concentrations. Conclusions. This nondestructive qCECT methodology calculated the depth-dependent GAG concentration for both native and trypsin-degraded cartilage at high spatial resolution. qCECT allows for more detailed understanding of the topography and depth dependency, which could help diagnose health, degradation, and repair of native and contrived cartilage.

Keywords
micro–computed tomography (μCT), contrast agent (Hexabrix [Ioxaglate]), glycosaminoglycans (GAG), cartilage, osteoarthritis (OA)

Introduction

Articular cartilage is comprised of an extracellular matrix (ECM) primarily composed of water, collagen fibers, and proteoglycans (PG) composed of negatively charged glycosaminoglycans (GAG). The interaction between these macromolecules creates a hydraulic permeability allowing diffusion from the synovial fluid to obtain nutrients essential to maintaining integrity of the tissue. The collagen fibers are oriented depth-dependently into 3 subtissue zones with respect to the surface defined as: parallel (superficial zone [SZ]), random (transitional zone [TZ]), and perpendicular (radial zone [RZ]). The collagen fibers in the ECM are intertwined with PG molecules depth-dependently in different concentrations. PG macromolecules are composed of a protein core composed of highly electronegative side chains, chondroitin and keratan sulfate, and hyaluronan, which binds aggrecan monomers to form highly charged aggregates that help regulate the diffusion of solutes attributed to the hydrophilic fixed charge density (FCD) and contributes to pore size with a link protein. Reduced GAG concentration, either natural or contrived, exhibits both an increased diffusion rate and increased equilibrium concentration of an anion due to the reduced FCD. Reduction of GAG alters the collagen fiber structural integrity and has been associated with the initial degradation and/or onset of diseases (e.g., osteoarthritis [OA]) and quantitative depth-dependent

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measurements may aid in diagnosing and treating non-
healthy cartilage.12,16,17

Electroneutrality of cartilage in a bathing solution of
ionic, anionic, or neutral molecules of different molecular
sizes has been investigated to determine the GAG concen-
tration using the Gibbs-Donnan theory.7,18,19 Microscopic
magnetic resonance imaging (µMRI) has been shown to
measure the depth-dependent FCD of cartilage attributed to
the GAG concentration, including delayed gadolinium-
enhanced MRI of cartilage (dGEMRIC), glycosaminogly-
cans chemical exchange saturation transfer, diffusion tensor
imaging, and sodium (23Na) imaging.20,24 The dGEMRIC
method calculates GAG based on cartilage in equilibrium
with a negatively charged paramagnetic contrast agent gad-
oinium diethylene triamine pentaacetic acid (Gd(DTPA)2−).20,25 T1 measurements before and after equil-
ibration with Gd can be used to calculate the GAG depth-
dependently based on Gibbs-Donnan equilibrium theory.

Recently, micro–computed tomography (µCT) has been used
to assess the integrity and predict changes in GAG
within healthy and degraded (e.g., biochemically and bio-
mechanically) articular cartilage using radiographic con-
trast agents of various molecular sizes and charge.10,14,26,36
Palmer et al.26 investigated the use of Hexabrix (Malinckrodt,
St. Louis, MO) equilibration in cartilage that could qualita-
tively predict differences in GAG content based on changes
in x-ray attenuation developing a technique called equilib-
rium partitioning of an ionic contrast agent via µCT. The
negatively charged Gd ions will diffuse depth-depend-
ently inversely related to the GAG in order to reach electro-
neutrality attributed to the repulsion of the negative charges.

The negatively charged Gd ions will diffuse depth-depend-
ently based on Gibbs-Donnan equilibrium theory. The
negatively charged Gd ions will diffuse depth-depend-
ently inversely related to the GAG in order to reach electro-
nutrality attributed to the repulsion of the negative charges.

The aim of the present study was to quantify and com-
pare of the depth and zonal dependencies of the GAG con-
centration of native and degraded ex vivo articular cartilage
using quantitative contrast enhanced micro–computed
tomography (µCECT) at high resolution.

Materials and Methods

Cartilage and Hexabrix Preparation

Fresh humeral heads were harvested, with the approval of
the local regulatory committee, within 5 hours of sacrifice
from three healthy and mature (~1-2 years old) canines that
were used in an unrelated scientific study. The load bearing
region of the humerus was divided into 3-mm sections
using a diamond saw (MTI Corporation, Richmond, CA)
and then sectioned into full-thickness rectangular blocks ~3
× 2 × 5 mm, which were immersed in 154 mM physiological
saline containing 1% protease inhibitor (S + PI) overnight
until experimentation (for a maximum of 4 days, from
the harvesting). Nineteen blocks were designated for no
treatment (native) and an additional 6 blocks were selected
for trypsin degradation (degraded). Trypsin (Sigma-Aldrich,
St. Louis, MO) solutions were prepared by adding 1.23 mg
of trypsin per 1 mL of S + PI. Full thickness cartilage-bone
specimens were immersed in trypsin for approximately 6
hours and then placed overnight in an S + PI bath to remove
any excess trypsin.

The cartilage contrast agent solution was prepared by
adding Hexabrix (Ioxaglate, Mallinckrodt Inc., St Louis,
MO) to S + PI in serial concentrations for use in the phan-
toms and bathing solutions. Hexabrix contains 320 mg/mL
of bound iodine and is an ionic dimer that is composed of 2
salts, namely, 393 mg/mL of ioxaglate meglumine and 196
mg/mL of ioxaglate sodium, which both dissociate into −1
charged ioxaglate with a molecular weight of 1269 g/mol.
In order to determine the relationship between the x-ray
attenuation and concentration of Hexabrix, Hexabrix phan-
toms (0%-100% in 10% increments including 5%) were
imaged to obtain a calibration curve for the x-ray
attenuation.

Micro–Computed Tomography Experiments

All µCT experiments were performed using Skyscan1174
(Bruker, Kontich, Belgium) with identical experimental
parameters: 40 kV, 110 mAs, 5 averages, 0.3° rotation step,
180° rotation, 0.2 mm Al filter, and 652 × 512 data matrix,
which took approximately 30 minutes to acquire approxi-
ately 608 images with a 13.4 μm isotropic voxel size. All
scans were reconstructed using NRecon (Bruker, Kontich,
Belgium) with the same parameters and a global intensity
threshold was kept constant for all scans to include the full
range of attenuation values. Figure 1A shows a representa-
tive 3-dimensional (3D) image of a native cartilage-bone
specimen equilibrated in Hexabrix.

Two-dimensional (2D) grayscale images were converted
to Hounsfield units (HU) based on the property that air =
−1000 HU and water = 0 HU.39 The conversion of linear
attenuation to HU was based on the calibration of air and
water:

\[
HU = 30.84 \times (\text{Gray value}) - 1000
\]  

(1)

Each cartilage block was gently blotted before the
experiments to remove any excess S + PI or Hexabrix and
secured in a radiopaque custom-made airtight holder con-
taining an S + PI soaked gauze to prevent evaporation.
during the experimentation. The cartilage blocks were manually aligned so that the articular surface was parallel to the direction of x-rays to minimize surface beam hardening. Each block was scanned after being immersed in S + PI to obtain a “baseline” scan (i.e., no Hexabrix), then immersed overnight (~24 hours) in a known Hexabrix bath concentration until equilibration then imaged again to measure the increase in x-ray attenuation. 

Preliminary investigations of eight native cartilage blocks were used to investigate Hexabrix bathing concentrations from 10% to 100% and found that 30%, 40%, and 50% obeyed the Gibbs-Donnan theory and took approximately 24 hours to reach equilibrium.14,29 Subsequently, 19 fresh native blocks were chosen to investigate Hexabrix solutions 30% (n = 5), 40% (n = 9), and 50% (n = 5) and 2 additional blocks per Hexabrix group were selected to investigate the effect of trypsin degradation.

**Image and Data Analysis**

Cartilage and phantom images were analyzed using ImageJ (National Institutes of Health, Bethesda, MD) and KaleidaGraph (Synergy, Reading, PA).40 Ten consecutive sagittal images near the central region of each phantom and cartilage block were averaged to improve the signal to noise ratio. A 20 × 20 pixel region of interest (ROI) was chosen for the phantoms to find the average attenuation for each Hexabrix concentration group. Figure 1A shows a representative 3D image of a cartilage block after equilibration in a 40% Hexabrix bathing solution. The image registration for the cartilage images before and after Hexabrix equilibration was determined manually by aligning both images in 3D space then finding similar landmark characteristics in the 2D sagittal images. A 20-column ROI, including the full-thickness of cartilage, was used to calculate the average depth-dependent attenuation at similar locations for each cartilage block before and after equilibration with Hexabrix as shown in Figure 1B. The average attenuation was calculated (mean ± standard deviation) at each depth to obtain a depth-dependent profile from the articular surface to the tissue-bone interface. The bulk GAG was defined as the full-thickness average. Zonal thicknesses were based on percentages depth-wise from the surface and the RZ is divided equally into 2 halves: 10% of cartilage is SZ, 10% is TZ, 40% is RZ1, and 40% is RZ2.41

The Hexabrix equilibrated cartilage HU profile was subtracted from the baseline HU profile to obtain the ΔHU depth-dependent profile. This value was then converted into the diffused concentrations of Hexabrix based on the calibration of the Hexabrix phantoms. The FCD and GAG depth-dependent profiles were calculated based on the Gibbs-Donnan equilibrium theory20,25:

\[
FCD = [\text{Na}^+]_b \times \left( \frac{[I]_t}{[I]_b} - \frac{[I]_b}{[I]_t} \right) \tag{2}
\]

\[
[GAG]_i = \frac{-502.5}{2} \times \frac{FCD}{502.5} \tag{3}
\]

where FCD is the fixed charge density, [Na\(^{+}\)]\(_b\) is the sodium ion concentration in the bathing solution; [I]\(_t\) and [I]\(_b\) are the Ioxaglate (Hexabrix) concentrations in the tissue and the bath, respectively, and [GAG]\(_i\) is the concentration of GAG in the tissue. The values “502.5” and “2” are constants attributed to 2 mol of negative charges for each mol of disaccharide with molecular weight of 502.5 g/mol.

**Statistical Model**

Using KaleidaGraph and JMP (SAS, Cary, NC), analysis of variance with Tukey’s post hoc test was used to determine (1) any effect of the Hexabrix bathing concentration on bulk GAG for native and/or degraded cartilage, (2) any effect on
bulk GAG from trypsin degradation compared with native cartilage, and (3) differences in zonal depth-dependent GAG concentrations for native and degraded cartilage. Significance was defined as $P < 0.05$, and any values $\leq 0.05$ showed a possibility for significance and indicated variation.

**Results**

In order to measure the concentration of Hexabrix based on x-ray attenuation from the μCT parameters, a calibration curve was determined by the average attenuation value from the Hexabrix phantoms and plotted in Figure 2. A positive linear relationship was found between the x-ray attenuation and Hexabrix concentrations up to 50% ($R = 0.997$):

$$y = 65.15 + 1900x$$

(4)

At higher concentrations, the x-ray attenuations of Hexabrix phantoms were no longer linear (data not shown). This slope of the linear calibration was used in the cartilage imaging experiments to convert x-ray attenuation to Hexabrix concentration, and used to calculate the $[I]$ in Equation (2) from the depth-dependent ΔHU profiles.

Two-dimensional representative sagittal images of native and degraded cartilage immersed in 30%, 40%, and 50% Hexabrix bathing solutions are shown in Figure 3. Morphologically, the lack of intensity variation across the width of all specimens indicates similar diffusion of the contrast agent at each specific depth of the tissue. In addition, small edge enhancement at the boundary of the tissue blocks was due to the beam-hardening effect and/or excess Hexabrix remaining after blotting. Intensity-wise, the degraded samples clearly show a marked increase in x-ray attenuation compared with the native cartilage regardless of bath concentration. The native cartilage was measured up to 500 μm and the degraded was measured up to 450 μm including the full-thickness, and the discrepancy is explained later in the Discussion section.

The depth-dependent profiles of cartilage equilibrated with Hexabrix (filled symbols) and “baseline” (open symbols) are plotted in Figure 4 for native and degraded cartilage. The “baseline” profiles do not show a marked difference either among Hexabrix bathing concentrations or between native and degraded cartilage. The depth-dependent HU profiles in both native and degraded cartilage (Fig. 4A and B) show increased attenuation for increasing Hexabrix bathing solutions (e.g., 30% < 40% < 50%). This increase in attenuation is consistent with cartilage having a larger influx of Hexabrix ions from an increased bathing concentration for native and degraded cartilage and is shown in Figure 4C and D, respectively.

Using Equations (2) to (4), the HU profiles were converted into the $[I]$ and FCD profiles (Fig. 4C-F), which represented the depth-dependent diffusion of Hexabrix (ioxaglate) into the tissue ($[I]$) at equilibrium and the FCD in the tissue. In native cartilage, the depth-dependent Hexabrix concentration of negative ions (Fig. 4C) shows an inverse relation to the FCD (Fig. 4E). In contrast, there was an increase in the depth-dependent Hexabrix concentration in degraded cartilage (Fig. 4D) that was related to the lower depth-dependent FCD (Fig. 4F). Finally, Figure 4G and H shows the GAG concentration when the cartilage was equilibrated in 30%, 40%, and 50% Hexabrix of healthy and degraded cartilage, respectively. The native cartilage shows a positive linear trend in the depth-dependent GAG measurements, and the degraded cartilage shows almost complete removal of GAG. The average bulk GAG for the native cartilage is 74.44 ± 6.09 mg/mL and for the trypsin-degraded samples is 11.99 ± 4.24 mg/mL. Wang et al., from our lab, used some of the adjacent humeri blocks to measure the GAG content using a T1 dGEMRIC protocol with various concentrations of Gd and found the bulk GAG to be 76.6 ±
3.3 and 16.2 ± 5.9 mg/mL for native and trypsin-degraded cartilage, respectively, and matched well with the GAG measurements from the inductively coupled plasma optical emission spectrometer (ICP-OES). The comparison between ex vivo experiments using μCT and μMRI were consistent and measured similar depth-dependent profiles of the GAG content of articular cartilage (data not shown).

Statistical analyses (P values) for the zonal GAG comparisons are shown in Table 1 and there was a demonstration of statistical significance for the comparisons. The
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native cartilage only demonstrated a statistical difference or variation when comparing GAG concentrations of 30% and 50% in RZ1 \((P = 0.0318)\) and RZ2 \((P = 0.0515)\) shown in Table 1a. The \(P\) value of 0.0515 is relatively close to the limit of \(P < 0.05\) and could show variation among the samples. GAG concentrations of degraded samples showed no statistical difference \((P > 0.7475)\) between various Hexabrix bathing concentrations shown in Table 1b. Comparisons between native and degraded GAG concentrations demonstrated statistical differences \((P < 0.032)\) for bulk, every zone, and every Hexabrix bath concentration shown in Table 1c.

### Discussion

In this study, we investigated the possibility to quantify the bulk and depth-dependent GAG concentrations from fresh (never frozen) \textit{ex vivo} native and degraded cartilage using \textit{q}CECT.\textsuperscript{33,44} The full-thickness bulk GAG of native cartilage was calculated to be \(74.44 \pm 6.09\) mg/mL and for the trypsin-degraded samples the GAG was \(11.99 \pm 4.24\) mg/mL, which are found to be consistent with the investigations in the literature and previous \(\mu\)MRI studies.\textsuperscript{19,20,25} There was no statistical difference in bulk GAG arising from the difference in Hexabrix bathing concentration, agreeing well with Silvast \textit{et al.}\textsuperscript{27} A statistical difference of GAG concentrations was demonstrated in the RZ of fresh native humeral cartilage when comparing Hexabrix bathing concentrations of 30% and 50%. The GAG difference in RZ1 could allow for a better understanding of the depth-dependent variations of healthy cartilage that may lead to better detection of degradation. The ability to detect depth-dependent changes in GAG concentration \textit{noninvasively} can only aid in the understanding of cartilage health, degradation, growth, and repair.\textsuperscript{4,5,15,30,34,41,45}

#### Hexabrix Phantoms

In order to relate Hexabrix diffusion in the tissue with attenuation, preliminary experiments were performed to optimize experimental parameters for Hexabrix attenuation in phantoms and native full-thickness cartilage. The slope calculated from the average Hexabrix phantom attenuation averages was shown in Equation (4) having a correlation of 0.997 demonstrating high accuracy and precision similar to calibrations found by Yoo \textit{et al.}\textsuperscript{14} who used both charged (Ioxaglate) and uncharged (Iopromide) contrast agents. Kallioniemi \textit{et al.}\textsuperscript{36} also found similar relationships between x-ray attenuation and contrast agent concentration using a contrast agent–enhanced peripheral quantitative computed tomography. These similar calibrations from both clinical and \(\mu\)CT resolutions allows for improved correlations and verifications using various contrast agents. These HU calibrations of Hexabrix concentration to attenuation values depend on equipment protocol, experimental settings, and reconstruction settings. This calibration is similar to the calibration of the \(R\)-value (i.e., diffusivity) used to calculate the GAG concentration by the \textit{d}GEMRIC method. Although there are different physical processes for the calibration of \textit{q}CECT and \textit{d}GEMRIC, the ability to have multidisciplinary quantitative correlations allows for increased verification and reproducibility of the GAG concentration in articular cartilage.\textsuperscript{17,20,46}

#### Table 1. ANOVA \(P\) Values for Bulk and Zonal GAG Comparisons.\textsuperscript{a}

| Zone | Native vs. Native | Degraded vs. Degraded | Native vs. Degraded |
|------|------------------|-----------------------|---------------------|
|      | Hex40% | Hex50% | Hex40% | Hex50% | Hex30% | Hex40% | Hex50% | Hex30% | Hex40% | Hex50% |
| Bulk | Hex30%  | 0.5189 | 0.3214 | Hex30%  | 0.9684 | 0.9225 | Hex30%  | <0.0001 | <0.0001 | <0.0001 |
|      | Hex40%  | 0.9897 | 0.9897 | Hex40%  | 0.9999 | 0.9999 | Hex40%  | <0.0001 | <0.0001 | <0.0001 |
| SZ   | Hex30%  | 0.999  | 1      | Hex30%  | 0.977  | 0.999  | Hex30%  | 0.005   | 0.005   | 0.005   |
|      | Hex40%  | 0.999  | 0.999  | Hex40%  | 0.999  | 0.999  | Hex40%  | 0.028   | 0.032   | 0.028   |
|      | Hex50%  | <0.0001| <0.0001| Hex50%  | 0.011  | 0.012  | Hex50%  | <0.0001 | <0.0001 | <0.0001 |
| TZ   | Hex30%  | 0.826  | 0.554  | Hex30%  | 0.994  | 0.998  | Hex30%  | 0.0005  | 0.0016  | 0.0107  |
|      | Hex40%  | 0.976  | 0.976  | Hex40%  | 0.999  | 1      | Hex40%  | 0.0004  | 0.0012  | 0.0085  |
|      | Hex50%  | <0.0001| <0.0001| Hex50%  | 0.0001 | 0.0001 | Hex50%  | <0.0001 | <0.0001 | <0.0001 |
| RZ1  | Hex30%  | 0.3791 | 0.0318 | Hex30%  | 0.9285 | 0.844  | Hex30%  | <0.0001 | <0.0001 | <0.0001 |
|      | Hex40%  | 0.4408 | 0.4408 | Hex40%  | 0.9999 | 1      | Hex40%  | <0.0001 | <0.0001 | <0.0001 |
| RZ2  | Hex30%  | 0.8496 | 0.0515 | Hex30%  | 0.961  | 0.7475 | Hex30%  | <0.0001 | <0.0001 | <0.0001 |
|      | Hex40%  | 0.2077 | 0.2077 | Hex40%  | 0.9931 | 0.9931 | Hex40%  | <0.0001 | <0.0001 | <0.0001 |
|      | Hex50%  | <0.0001| <0.0001| Hex50%  | <0.0001| <0.0001| Hex50%  | <0.0001 | <0.0001 | <0.0001 |

\(\text{ANOVA} = \text{analysis of variance}; \text{GAG} = \text{glycosaminoglycan}; \text{Hex} = \text{Hexabrix}; \text{RZ} = \text{radial zone}; \text{SZ} = \text{superficial zone}; \text{TZ} = \text{transitional zone}.\)

\(\text{The values in boldface highlight the variation found in native cartilage between Hexabrix bathing concentrations of 30\% and 50\%}.\)
**Bulk GAG Measurements Using qCECT**

qCECT was used to quantitatively measure the x-ray attenuation and to calculate the GAG concentration in native and degraded articular cartilage in equilibrium with a negatively charged contrast agent (Hexabrix). The equilibration was found to follow Gibbs-Donnan equilibrium theory and the bulk GAG concentration was calculated to be 74.44 ± 6.09 and 11.99 ± 4.24 mg/mL for native and degraded cartilage, respectively. This measured difference is attributed to a decreased GAG concentration attributed to the degradation from trypsin. Yoo et al. investigated the depth-dependent protein concentrations of human tibial cartilage using mass spectroscopy and found aggrecan and link protein had a linear increasing concentration with depth and other depth-dependent proteins that could affect diffusion in different zones. The linear increase of aggrecan is consistent with the increasing FCD found in this study. Although qCECT cannot distinguish individual types of proteins, the interactions between Hexabrix and various proteins may be the reason for the diffusion difference in the RZ of fresh native cartilage. The detection of these subtle changes attributed to tissue integrity allow for future investigations into the detection and clearer understanding of tissue health and degradation.

**Depth Dependency**

A clearer understanding of the depth dependency of articular cartilage is crucial in determining health and the alterations that occur from degradation, disease, or injury (e.g., OA, aging, sports injuries, etc.) and monitoring the efficacy of repair and regrowth. The statistical difference demonstrated between native and degraded cartilage allows for the possibility of qCECT to detect depth-dependent changes from damage (e.g., from aging) or disease (e.g., OA). The depth-dependent diffusion and equilibration of various Hexabrix bathing concentrations shows the effects that are attributed to the FCD, pore size, collagen cross-linking, and steric interactions. Kokkonen et al. investigated the diffusion flux and equilibration and found that the main reason for the diffusion of ioxaglate and iodide was attributed to the FCD and investigated the effects of cross-linking and steric interactions. They showed that the FCD has a greater effect on the equilibration than the cross-links; however, the collagen cross-linking is important to cartilage integrity.

In this current study, the deep tissue subzones RZ1 and RZ2 demonstrated a statistical difference in GAG concentration of fresh native articular cartilage when comparing 30% and 50% Hexabrix bathing concentrations. Since there was no other effect from Hexabrix concentration in the zonal GAG measurements, the difference in the RZ could be attributed to the variation of the tissue properties. Müller et al. investigated the depth-dependent protein concentrations of human tibial cartilage using mass spectroscopy and found aggrecan and link protein had a linear increasing concentration with depth and other depth-dependent proteins that could affect diffusion in different zones.

**Micro–Computed Tomography Comparisons**

The ability to quantify the depth-dependent GAG concentration will allow μCT to have quantitative comparisons with other techniques. Silvast et al. investigated the diffusion and partition function of ioxaglate and iodide in cartilage plugs from the patella and found no difference in attenuation among bathing concentrations. The investigation of the partition function is important for in vivo cartilage experiments since equilibration is not required and the contrast agent diffusion rate is related to the GAG concentration. Bansal et al. investigated the electrostatic attraction of 2 cationic (CA1+ and CA4+) contrast agents and electrostatic repulsion of 1 anionic (CC2) contrast agent and found that the cations provided a more accurate prediction of the GAG concentration. The ability to use positive, neutral, and negative charged particles allows for a better understanding of the diffusion and equilibration in cartilage related to the overall tissue properties. The use of positively charged contrast agents could be used to investigate the variation of GAG we found in the RZ of fresh native cartilage because of the limited diffusion in the deep zones of cartilage.

**Cartilage Thickness**

The fresh native cartilage was measured up to 500 μm, which includes the full-thickness, and the degraded tissue was measured up to 450 μm. Trypsin can cause cartilage to shrink after degradation, and the “baseline” and Hexabrix cartilage images did not show much observable difference in full-thickness measurements as well as initial thickness measurements before each scan. Torzilli et al. reported that the hydrophilic PG molecules are restrained from expanding by the tensile strength of the collagen fiber network. This restraint as well as the removal of the hydrophilic PG could be possible causation of the 50 μm differences of the full-thickness observed in this study.

**Experimental Limitations**

There were several limitations of this study. This study used a relatively low sample size; however, the statistical analyses demonstrated high significance, and comparable results were similar to the other studies in the literature. The requirements for Gibbs-Donnan equilibrium were not
satisfied when there was a large mismatch in osmolarities between cartilage and bathing solution and were not further investigated. There may have been a slight beam hardening effect because no algorithm was used for mathematical correction; however, the sample was aligned to minimize the beam hardening effect on the articular surface. Also, the trypsin-degraded samples should have an increased beam hardening effect, which was not observed. This quantitative imaging method using μCT is currently limited to ex vivo samples, because of the requirement of Hexabrix equilibration that may not possible to obtain for in vivo cartilage. This study used only one form of degradation and one negatively charged radiographic contrast agent. However, to the best of our knowledge, this is the first μCT study to quantitatively measure the depth-dependent GAG concentration using Gibbs-Donnan equilibrium and investigations within the subtissue zones of articular cartilage. Finally, we did not carry out the histological assay for GAG determination in this project. However, the specimens used in this project came from the same tissue source (the same age animals of the same species) for our laboratory for more than 12 years. We have used these specimens in various imaging and non-imaging projects, many quantitative comparisons among multidisciplinary techniques. For example, Wang et al.\(^{42}\) compared microscopic MRI and ICP-OES quantification using the nearly identical tissue. The measurement from μCT in this project was in excellent agreement with all of our previous quantitative measurements.

**Conclusions**

In conclusion, CT has recently become an invaluable tool for monitoring the integrity for both in vivo and ex vivo cartilage.\(^{15,16,33,37}\) qCECT was used in this project for the first time to quantify the depth-dependent GAG concentration and investigate the zonal variations from both healthy and degraded cartilage. The ability to quantify the depth-dependent concentration of GAG molecules in cartilage will allow possibilities for a better understanding and detection of disease and degradation. High-resolution (e.g., 13.4 μm\(^3\)) studies are helpful in detecting differences in subtissue zones and have the possibility of being applied to lower resolution (e.g., 0.200 mm\(^3\)) images to help diagnose topological differences in cartilage health, degradation, and repair.\(^{51}\) The ability to quantitatively compare cartilage integrity using qCECT could allow for better comparisons of multidisciplinary investigations, which may allow for a more detailed understanding of in vivo, ex vivo, and in situ cartilage.

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**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the authorship and/or publication of this article.

**Ethical Approval**

This study was approved by our local institutional review board, IACUC.

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