Contribution of Proteoglycan Osmotic Swelling Pressure to the Compressive Properties of Articular Cartilage

EunHee Han, Silvia S. Chen, Stephen M. Klisch, and Robert L. Sah

ABSTRACT  The negatively charged proteoglycans (PG) provide compressive resistance to articular cartilage by means of their fixed charge density (FCD) and high osmotic pressure (πPG), and the collagen network (CN) provides the restraining forces to counterbalance πPG. Our objectives in this work were to: 1), account for collagen intrafibrillar water when transforming biochemical measurements into a FCD-πPG relationship; 2), compute πPG and CN contributions to the compressive behavior of full-thickness cartilage during bovine growth (fetal, calf, and adult) and human adult aging (young and old); and 3), predict the effect of depth from the articular surface on πPG in human aging. Extrafibrillar FCD (FCD_{EF}) and πPG increased with bovine growth due to an increase in CN concentration, whereas PG concentration was steady. This maturation-related increase was amplified by compression. With normal human aging, FCD_{EF} and πPG decreased. The πPG values were close to equilibrium stress (τEQ) in all bovine and young human cartilage, but were only approximately half of τEQ in old human cartilage. Depth-related variations in the strain, FCD_{EF}, πPG, and CN stress profiles in human cartilage suggested a functional deterioration of the superficial layer with aging. These results suggest the utility of the FCD-πPG relationship for elucidating the contribution of matrix macromolecules to the biomechanical properties of cartilage.

INTRODUCTION

The main extracellular matrix components of articular cartilage, proteoglycans (PG) and collagens (COL), provide biomechanical properties that vary with growth, aging, and depth from the articular surface. The negatively charged PG contribute to compressive resistance and provide a high osmotic pressure (πPG) within the tissue. In contrast, the collagen network (CN) provides the restraining stress that counterbalances πPG at rest or during loading (Fig. 1, A and B), and the high resistance of cartilage to tension (1). Nearly 90% of PG is aggrecan, which complexes with hyaluronic (HA) and link protein to form large PG aggregates entrapped within the CN (2). The aggrecan monomers contain many long chains of sulfated glycosaminoglycans (GAG), specifically chondroitin sulfate (CS) and keratan sulfate (KS). CS and KS, in turn, provide a fixed charged density (FCD) to the tissue due to the sulfate and carboxyl groups of CS and sulfate groups of KS. With the electrostatic repulsion of negatively charged GAG moieties, the FCD contributes to the compressive resistance of the tissue by providing πPG within the tissue (3).

Native cartilage tissue exhibits variations in compressive properties, both with age and with depth from the articular surface, due in part to variations in biochemical content. Even with a steady GAG concentration during bovine growth from fetal to adult stages, cartilage compressive modulus increases ~2-fold due an increase in COL concentration by two- to threefold (4). On the other hand, human aging is associated with a trend of decreasing cartilage compressive modulus and PG concentration while COL concentration remains steady (5,6). Cartilage also displays compressive properties and biochemical content and organization that vary with depth from the articular surface to the bone. The superficial layers have a relatively low FCD and exhibit larger strains during compression compared with the deeper layers (7–9). The depth-dependent variations in water content, PG content, FCD, and πPG seen in normal cartilage are altered with osteoarthritic disease in association with aging (7). Such alterations perturb the balance between PG swelling and CN restraining stress, and the normal mechanics of the tissue (1).

Several models have been proposed to describe the relationship between πPG and PG content, usually expressed via FCD or GAG concentrations (10–13). The πPG contribution to overall cartilage mechanical properties, such as compressive modulus or aggregate modulus, has been estimated (10,12,14). Using the concept of balance of forces with πPG, investigators have also estimated the CN contribution to compressive resistance (3,11,15). Such comparisons are affected by the accuracy of FCD and πPG calculations, and assumptions made in those calculations. Estimates of FCD imply but often do not account explicitly for CS and KS charge differences (z_{CS} = -2, z_{KS} = -1 pr disaccharide) and varying CS/KS ratios, which vary substantially with age and depth from the articular surface (7,16). Additionally, because πPG models are typically developed from relationships with aggrecan or CS in solution, investigators have not needed to consider the interaction between PG and COL. However, it has been proposed that in articular cartilage, water is distributed between COL fibrils (intrafibrillar (IF)) and PG (extrafibrillar (EF); Fig. 1, C and E), and this water distribution varies with external stress applied to the
tissue (11,17). The fluid shift from IF to EF with applied stress has been estimated from the lateral spacing of COL fibrils within cartilage (Fig. 1, D and F) as determined from x-ray scattering experimental data and previously described models (i.e., the Ogston and Hodge-Petruska models) (17,18). Thus, the effective FCD and associated $\pi_{\text{PG}}$ in cartilage may be higher than apparent values and need to be calculated based on EF water. Such modulation of $\pi_{\text{PG}}$ by the CN may affect the biomechanical properties of cartilage.

FCD-$\pi_{\text{PG}}$ models may provide a useful tool for elucidating the relationship between the composition and function of articular cartilage, because they allow estimation of $\pi_{\text{EG}}$ from a known PG concentration or FCD. Combined with measurements of tissue mechanical properties, these models can also provide insights into the CN’s mechanical properties (19). The effect of compression on FCD and $\pi_{\text{PG}}$ was previously considered for full-thickness cartilage (10) and layers of adult bovine cartilage (20), but has not been characterized with growth and aging. Thus, our objectives in this work were to 1) describe relationships to explicitly account for variations in CS/KS ratios and exclusion of IF water in a FCD-$\pi_{\text{PG}}$ relationship; 2) predict $\pi_{\text{PG}}$ and COL contributions to compression using experimentally obtained biochemical data and compressive equilibrium stress ($\sigma_{\text{EQ}}$) for full-thickness cartilage at various stages of bovine growth (fetal, calf, and adult) and human aging (young and old); and 3), predict the effect of depth from the articular surface in human young and old cartilage on $\pi_{\text{PG}}$ using experimentally obtained biochemical data.

**METHODS**

**Bovine cartilage biochemical and biomechanical data**

For the bovine cartilage studies, we used data from a previous study (4). Briefly, 1000-µm-thick cylindrical slices ($d = 4.8$ mm) were taken from bovine fetal (2nd and 3rd trimester, $n = 6$), calf (1–3 months old, $n = 7$), and adult (1–2 years old, $n = 7$) cartilage from a site-matched, central region of a femoral condyle. The samples were analyzed for wet weight (WW), equilibrium stress after uniaxial confined compression to compressive strain (t) of 15% and 30%, dry weight (DW), sGAG content by dimethylmethylen blue (DMMB) assay (21), and COL content by hydroxyproline assay (22) (Fig. S1). Samples from the patella-femoral groove cartilage were similarly tested, and the results are presented in the Supporting Material.

**Human cartilage biochemical and biomechanical data**

For the human cartilage studies, we used data from a previous study (23). Briefly, normal adult human articular cartilage from a site-matched, antero-medial region of femoral condyles from young (30 ± 2 years old, $n = 7$) and old (69 ± 2 years old, $n = 7$) cadaveric donors were analyzed. Spanning the majority of the thickness from the surface to the deep zone, ~250-µm-thick slices of the tissue were each analyzed for WW, DW, sGAG content, and COL content (Fig. S1). Donor-matched hemicylindrical osteochondral samples with full-thickness articular cartilage ($d = 4.8$ mm) were subjected to uniaxial confined compression to an overall compression of ~10%, ~20%, and ~30% (24,25). At equilibrium, the stress was measured along with the depth-dependent displacement and strain (23).

**Incorporation of the CS/KS ratio and exclusion of IF water into the FCD-$\pi_{\text{PG}}$ relationship**

To compute FCD, accounting for variation in the CS/KS ratio, we described a relationship (Eq. 1) using the molecular mass (MW) per disaccharide of CS (MWCS = 457 g/mol) and KS (MWKS = 444 g/mol), masses of CS and KS ($m_{\text{CS}}$ and $m_{\text{KS}}$), mol-charges of CS and KS ($z_{\text{CS}} = 2$ and $z_{\text{KS}} = 1$ charge/disaccharide), and mass of EF water ($m_{\text{EF,1H2O}}$). We calculated the MW of CS and KS from the molecular structures of each disaccharide found in the repeating portion of a chain. CS and KS content can be determined by several methods, including enzyme-linked immunosorbent assay, selective enzymatic digestion, and assays that take advantage of different hexose compositions of CS and KS (26–29). Details of the FCD$_{\text{EF}}$ calculation from commonly used assays to determine GAG content are provided in the Supporting Material.

\[
FCD_{\text{EF}} = \left(\frac{m_{\text{CS}} \times z_{\text{CS}}}{MW_{\text{CS}}}\right) + \left(\frac{m_{\text{KS}} \times z_{\text{KS}}}{MW_{\text{KS}}}\right) \times \frac{m_{\text{EF,1H2O}}}{1000 \text{mEq charge}}.
\] (1)

To describe $\pi_{\text{PG}}$ based on FCD$_{\text{EF}}$, we fit a piecewise continuous function of four segments with monotonically increasing, quadratic equations and continuous first derivatives to the FCD-$\pi_{\text{PG}}$ data by weighted least-squared error fit (Fig. 2). The PG-$\pi_{\text{PG}}$ data from Fig. 2 of Buschmann and...
Grodzinsky (10), originally from Williams and Comper (30), and FCD-τPG data from Fig. 3 of Basser et al. (11), originally from Urban et al. (31), were used for the fit that was made to be continuous to FCD-τPG points from the Donnan equations at FCD > 0.5 mEq/ml (10). The Donnan model provides a good model at higher FCD or under macro-continuum conditions because the Donnan and Poisson-Boltzmann-cell models converge under those conditions (32), and both fit the high FCD-τPG data from Basser et al. (11). We converted the PG concentrations from Buschman and Grodzinsky (10) to FCD using DW/uronic acid = 3.29 (for KS-free rat chondrosa (downward tic marks) for the corresponding FCD (upward tic marks) compartments: 

\[ M_{\text{glucuronolactone}} = 176.124 \text{ g/mol} \] (33) as described in the Supporting Material.

The four-segment piecewise continuous equations to describe the FCD-τPG relationship were of the form

\[ \pi_{\text{PG},i} = a_i (\text{FCD}_{\text{EF}} - x_i)^2 + b_i (\text{FCD}_{\text{EF}} - x_i) + c_i \text{ for } x_i < \text{FCD}_{\text{EF}} \leq x_{i+1} \] (2)

with the constants in Table 1. This FCD-τPG relationship provided a good fit, including at low FCD values, which are typical of cartilage in the superficial zone and at low compression (Fig. 2).

For samples of cartilage, where WW, DW, fixed charge mass (i.e., in Eq. 1), and COL mass (m_{COL}) are given (e.g., determined experimentally), the EF FCD (FCD_{EF}), and consequently ρ_{PG}, from Eq. 2 can be calculated (11) as follows:

The total water content (m_{H2O}) is the difference between WW and DW:

\[ m_{\text{H2O}} = \text{wet weight} - \text{dry weight}. \] (3)

The fluid mass, m_{H2O}, is distributed between EF (m_{EF,H2O}) and IF (m_{IF,H2O}) compartments:

\[ m_{\text{EF,H2O}} = m_{\text{H2O}} - m_{\text{IF,H2O}}. \] (4)

The fluid content of COL, m_{IF,H2O}, has been determined experimentally to be related to EF stress (τ_{EF}), where τ_{EF} = τ_{PG}, by the following expression (11,34):

\[ m_{\text{IF,H2O}} = (0.726 + 0.538 \times \exp(-0.258 \times \tau_{\text{EF}})) \times m_{\text{COL}}. \] (5)

Because Eqs. 1, 2, 4, and 5 are coupled and do not have an explicit solution, the four unknowns, m_{EF,H2O}, m_{IF,H2O}, FCD_{EF} and τ_{PG}, are calculated iteratively until τ_{EF} and τ_{PG} converge (11). Conceptually, when fluid is distributed appropriately between m_{EF,H2O} and m_{IF,H2O}, for the charge and COL present, τ_{PG} of Eq. 2 just balances the τ_{EF} of Eq. 5.

### τ_{PG} and σ_{COL} for various stages of growth and aging under compression

We studied the effect of IF water exclusion by calculating FCD normalized by total water content or by EF water content and the resulting τ_{PG} for bovine fetal, calf, and adult femoral condyle cartilage under compression of 0–30%.

Using the FCD-τ_{PG} relationship described above, we estimated τ_{PG}-values during compression for full-thickness cartilage using the experimentally obtained biochemical data for various stages of bovine growth (fetal, calf, and adult) (4) and human aging (young and old; Fig. S1) (23).

For human cartilage, we computed thickness-weighted averages of the biochemical data to obtain biochemical values for the full-thickness tissue. Then, for both bovine and human cartilage, we determined FCD_{PG} from the total CS-equivalent sGAG content measured using the DMMB assay with CS sodium salt (Sigma, St. Louis, MO) standards, accounting for the presence of impurities such as water and extra sodium salts (~14–15%). Whereas the CS and KS contents were not separately measured, the measured CS-equivalent sGAG content from the charge-based DMMB assay was directly converted to FCD. Details are provided in the Supporting Material.

To estimate τ_{PG} for each sample under compression, we first calculated FCD_{EF} at each compression level. With compression, we assumed that matrix m_{AG} and m_{COL} was maintained in the tissue while fluid was expelled as displaced volume (ΔV). The relationship for EF water with compression is

\[ m_{\text{EF,H2O}} = m_{\text{H2O}} - m_{\text{IF,H2O}} - \rho_{\text{water}} \times \Delta V \] (6)

where \( \rho_{\text{water}} = 1.0 \) and \( \Delta V = \varepsilon \pi r^2 \) for a cylindrical sample or \( \Delta V = \varepsilon \pi r^2 \) for a hemicylindrical sample under uniaxial confined compression of ε. Then, we determined FCD_{EF}, τ_{PG}, m_{EF,H2O}, and m_{IF,H2O} using the FCD_{EF}–τ_{PG} model as described above.

We estimated the CN contribution to compression, CN stress (σ_{CN}), at each compression level, from the calculated τ_{PG} and experimentally obtained compressive equilibrium stress (σ_{EQ}) using the balance of forces (3,11):

\[ \sigma_{\text{EQ}} = \sigma_{\text{PG}} + \sigma_{\text{CN}}. \] (7)

In Eq. 7, each component (τ_{PG} and σ_{CN}) contributes stress to the overall tissue volume. The τ_{PG} is stress that is generated by PG (Eq. 2) but affects the tissue throughout via compaction of CN (Eq. 5). Similarly, the σ_{CN} is counterbalancing stress of the CN, considering the entire tissue.

We then calculated the CN prestress at 0% compression level and compression level at σ_{CN} = 0 kPa for both bovine and human cartilage. For compression level at σ_{CN} = 0 kPa, only samples in which σ_{CN} transitioned from tension (negative value) to compression (positive value) were considered (bovine: n = 4–6; human: n = 6).
\( \pi_{PG} \) with depth and age in human cartilage under compression

The \( \sigma_{CDEF}, \pi_{PG}, \) and \( \sigma_{CN} \) were calculated in 10 normalized layers through the thickness of young and old human cartilage. To estimate \( \pi_{PG} \) for \( \sim 250\mu \text{m} \)-thick layer \( i \), we first calculated free EF water \( (m_{EF, CDEF,i}) \) from Eq. 4 using experimentally obtained strains \( (\varepsilon_i) \) for each layer at each overall compression levels of \( -10\%, -20\%, \) and \( -30\% \) (23). We then calculated the FCD\(_{EF} \), based on biochemical data for each layer, and calculated \( \pi_{PG} \) for each layer throughout full-thickness cartilage using the FCD-\( \pi_{PG} \) fit (Eq. 2). We estimated the \( \sigma_{CN,i} \) in each layer at each strain from the calculated \( \pi_{PG} \) and \( \sigma_{EQ} \) using Eq. 7. Then, the weighted averages of \( \varepsilon_i \), FCD\(_{EF,i} \), \( \pi_{PG,i} \), \( \sigma_{CN,i} \), total water/WW, and EF water/WW were calculated for each of 10 layers through the depth of human cartilage; layer 1 was the most superficial layer at the articular surface, and layer 10 was the deepest layer next to the subchondral bone.

Statistical analysis

Data are presented as the mean \( \pm \) standard error. The effect of bovine growth on cartilage biochemical data, and FCD\(_{EF} \) and \( \pi_{PG} \) at each compression level was assessed by one-way analysis of variance (ANOVA) and Tukey's post-hoc test. For human cartilage, the effect of aging was assessed by repeated-measures ANOVA with the depth as a repeated factor at each compression level. When the age had a significant \( (p < 0.05) \) independent or interactive effect with layer, each layer was analyzed separately. When the depth had a significant effect \( (p < 0.05) \), pairwise comparisons of layers for either young or old cartilage were performed with a Sidak correction of the \( p \)-value.

RESULTS

Variation of CS/KS ratios and IF water exclusion were incorporated into the calculation of FCD (Eqs. 1–6). Approximately twice the mass of KS relative to CS was equivalent to the same FCD (Fig. 2 C). Also, \( \pi_{PG} \) increased with an increasing CS/KS ratio, reflecting the charge difference between KS and CS. Considering IF water and using only EF water for calculation of PG-associated properties in (Eqs. 4–6), FCD and, as a result, \( \pi_{PG} \) were substantially higher than values calculated using total water content for cartilage. With compression, the differences in FCD and \( \pi_{PG} \) calculated with EF water instead of total water content became even more pronounced (Fig. 3).

Applying the FCD\(_{EF} \)-\( \pi_{PG} \) relationship to data from full-thickness bovine femoral condyle cartilage revealed that FCD\(_{EF} \) and \( \pi_{PG} \) changed with growth (ANOVA, \( p < 0.05 \) for FCD\(_{EF} \) at 0, 15\%, and 30\%, and \( \pi_{PG} \) at 30\% compression; Figs. 4 A and 5). Calf and adult femoral condyle cartilage generally had higher FCD\(_{EF} \) and \( \pi_{PG} \) values than fetal cartilage at each compression level \( (p < 0.05 \) for calf versus fetal for FCD\(_{EF} \) at 0–30\%, and \( \pi_{PG} \) at 30\% compression). Even with similar GAG/WW at zero strain, the higher FCD\(_{EF} \) in calf and adult cartilage was due to higher COL content (Fig. S1) and increased IF water (Fig. 3). For bovine cartilage, \( \pi_{PG} \) closely approximated \( \sigma_{EQ} \) for all growth stages at all compression levels (Fig. 5). The \( \sigma_{CN} \) were generally low and moved from tension (negative in this convention) at the reference state to compression (positive stress) with increasing applied compression. For full-thickness adult human cartilage, young cartilage had higher FCD\(_{EF} \) and \( \pi_{PG} \) than old cartilage at all compressive strains \( (p < 0.01; \) Figs. 4 B and E). The \( \pi_{PG} \) for young cartilage closely approximated \( \sigma_{EQ} \) at all strain levels, whereas \( \pi_{PG} \) for old cartilage accounted for only approximately half of \( \sigma_{EQ} \) (Fig. 6). The low \( \pi_{PG} \) for old cartilage suggested a larger proportion of \( \sigma_{CN} \) contribution to \( \sigma_{EQ} \) than was found in young human cartilage. The \( \sigma_{CN} \) for both young and old cartilage generally increased with compressive strain, moving from tension toward compression.

The properties of CN under compression were altered with growth and aging of cartilage. The CN prestress for bovine calf and adult cartilage tended to be higher than that for fetal cartilage (ANOVA \( p = 0.19; p = 0.20 \) for adult and \( p = 0.28 \) for calf versus fetal; Fig. 7 A). In human cartilage, young cartilage had higher CN prestress than old cartilage \( (p < 0.01; \) Fig. 7 B). The compression level at \( \sigma_{CN} = 0 \), changing from prestressed tension to compression, tended to be higher for bovine calf and adult cartilage than for fetal cartilage (ANOVA \( p = 0.24; p = 0.38 \) for adult and \( p = 0.28 \) for calf versus fetal; Fig. 7 C) and for human young cartilage than for old cartilage \( (p = 0.20; \) Fig. 7 D).
Under compression, the profiles of strain, FCD$_{EF}$, $\pi_{PG}$, $\sigma_{CN}$, total water content, and EF water content for human cartilage varied with depth from the articular surface (ANOVA $p < 0.001$ for strain and EF water at all compressions, and for FCD$_{EF}$, $\pi_{PG}$, $\sigma_{CN}$, and total water content at 0, 20%, and 30% compression; Fig. 8, Fig. S4, and Fig. S5). These profiles also were significantly different with the tissue age alone ($p < 0.01$ for FCD$_{EF}$ and $\pi_{PG}$ at all compressions, and for $\sigma_{CN}$ at 0% compression) and interactively with depth ($p < 0.01$ for FCD$_{EF}$, $\pi_{PG}$, and $\sigma_{CN}$ at 0% compression and for strain at 10% compression).

The strain profiles in young and old cartilage were distinct from each other. The highest compressive strains in young cartilage were found only in the most superficial layer and linearly decreased with depth at all compression levels ($p < 0.05$ for layer 1 versus 5–10 at 20% compression; Fig. 8, A and C–E). However, in old cartilage, the highest strains were more evenly distributed into the middle layer, and strain then decreased through the depth of the cartilage ($p = 0.054$ for layer 1 versus 9 and 10; $p < 0.05$ for layer 2 versus 8–10 at 20% compression; Fig. 8, B–E).

The FCD$_{EF}$ and $\pi_{PG}$ profiles differed with depth ($p < 0.001$ at 0, 20%, and 30% compression) and between old and young cartilage ($p < 0.005$; Fig. 8, F–K, and Fig. S3). At zero strain, the local FCD$_{EF}$ and $\pi_{PG}$ varied with depth ($p < 0.001$) and aging ($p < 0.001$; Fig. 8, F–K). The superficial layers had lower FCD$_{EF}$ and $\pi_{PG}$ than the deeper layers in both young and old cartilage (e.g., $p \leq 0.05$ for layer 1 versus 6), with young cartilage having higher FCD$_{EF}$ and $\pi_{PG}$ in deeper layers than old cartilage ($p < 0.05$ for layers 5–10). With compression, FCD$_{EF}$ and $\pi_{PG}$ profiles for young cartilage increased in the superficial layers, evened out through the depth of the cartilage at 10% and 20% compression ($p > 0.2$), and peaked in the superficial layers and the upper deep layers at 30% compression ($p < 0.05$ for layer 5 versus layers 9 and 10). However, FCD$_{EF}$ and $\pi_{PG}$ profiles for old cartilage tended to peak in the middle layer (layer 4) at all compression levels, and showed increasing amplitude with increasing compression.

For both young and old cartilage, the $\sigma_{CN}$ profiles at zero compression were generally in tension more in the deep layer than in the superficial layer ($p < 0.05$ in young and $p = 0.056$ in old for layer 1 versus 6; Fig. 8, L–Q). The $\sigma_{CN}$ profiles shifted toward compression at 10% and 20% compression, with old cartilage tending to shift to slightly higher stresses than young cartilage at corresponding depth layers (Fig. 8, O and P). At 30% compression for young cartilage, superficial and middle-layer CN were back in tension, whereas the deep-layer CN was in compression (Fig. 8 Q). For old cartilage, most of the CN was in compression with just the middle layers in tension.

The FCD$_{EF}$, $\pi_{PG}$, and $\sigma_{CN}$ profiles for old cartilage generally were similar to the trends in the profiles for young cartilage at a normalized depth of $\geq 0.2$. These variations in FCD$_{EF}$, $\pi_{PG}$, and $\sigma_{CN}$ with depth of cartilage and aging may play a role in the changes observed with the overall mechanical properties of the tissue.

**DISCUSSION**

In this work, we applied the FCD$_{EF}$–$\pi_{PG}$ relationship to predict the contribution of PG to the compressive properties of articular cartilage. By accounting for sGAG content more accurately and for exclusion of IF water to PG, this
approach appears to predict $\pi_{PG}$ reasonably well for both bovine and human cartilage at various stages of growth and aging, and with depth from the articular surface during compression. Even with similar GAG/WW, more-mature cartilage (bovine calf and adult) had higher FCD$_{EF}$ and $\pi_{PG}$ than less-mature tissue (bovine fetal), and this effect was amplified with compression. With aging, the overall FCD$_{EF}$ and $\pi_{PG}$ were lower in old human cartilage as compared with young cartilage. The $\pi_{PG}$-values were close to $\sigma_{EQ}$ in bovine cartilage with growth and in human young cartilage, but only approximately half of $\sigma_{EQ}$ in human old cartilage. The strain, FCD$_{EF}$, $\pi_{PG}$, and $\sigma_{CN}$ profiles revealed depth-related variations in human cartilage that were substantially altered with normal aging, suggesting deterioration of a functional superficial layer. These results demonstrate that the FCD$_{EF}$--$\pi_{PG}$ relationship elucidated here can provide a useful tool for assessing the contribution of PG and its interaction with the CN to the biomechanical properties of cartilage as they vary with growth, aging, and depth from the articular surface.

For a precise calculation of $\pi_{PG}$, it is important to obtain an accurate estimate of FCD$_{EF}$ from the total sGAG content. The CS/KS ratio varies across joint surfaces (35), decreases with growth and depth (26), and increases with degeneration of articular cartilage (16), and the charge difference between CS and KS can affect FCD$_{EF}$ determined from GAG mass by as much as 50%. Nonsulfation or double sulfation of the GAG was not taken into account in the FCD equation presented here, because previous studies indicated that the assumption of normal sulfation gave excellent agreement between calculated and experimentally measured FCDs (35). The accurate accounting of MW in converting the mass of CS and KS into FCD is important because values in literature vary by as much as $\pm$10% (457 g/mol versus 503 g/mol for CS disaccharide (35)). Here, 457 g/mol disaccharide was chosen with the assumption of CS in a long chain, with loss of a water molecule between 2 disaccharides due to a glycosidic linkage (see Supporting Material for details).

The curve fit of the FCD--$\pi_{PG}$ relationship appears to provide good estimates of $\pi_{PG}$, especially at low FCD$_{EF}$ as found in cartilage in the superficial zone and at low compression. Previous FCD--$\pi_{PG}$ fits, such as the quadratic relationships presented in works by Bassere et al. (11) and Chahine et al. (12), provide excellent fits for FCD > 0.16 mEq/ml. To extend the FCD--$\pi_{PG}$ relationship to the lower FCD range that is important for bovine cartilage and the upper layers of human articular cartilage, we fit a piecewise quadratic relationship to experimental data (10,11), including the low-FCD data from Williams and Comper (30). Although the experimental data were obtained from an extracted aggrecan solution, the measured FCD$_{EF}$--$\pi_{PG}$ relationships were similar for extracted aggrecan in solution or for an intact tissue from the intervertebral disc (36). The FCD--$\pi_{PG}$ data points and the experimental data used here were for samples in a bath solution with isotonic buffer or equivalent to 0.15 M NaCl, which is typical of the environment within a joint. The curve-fitting approach used here may also be useful for describing FCD--$\pi_{PG}$ relationships at other salt concentrations from experimental data.

In our analysis of cartilage, we assumed the presence of COL hydration (IF water) and the unavailability of water...
(while in the IF space) to PG or other surrounding larger molecules, as studied previously (17,18,37). Because aggregan monomers are impermeable to membranes with pore sizes < 125 nm (38), and the IF space, at least on the outer surface of the COL fibril, is no larger than the gap region on the COL fibril or approximately half of a period (~34 nm) (39), the large polyanionic PG are unlikely to exchange into the IF space. The deformation of COL fibrils due to $\pi_{\text{PG}}$ (17,18) implies a molecular-level balance of stresses between the EF and IF spaces. Thus, $\pi_{\text{PG}}$ represents the swelling tendency of aggregan in the EF space and, equally, the counterbalancing resilience of the CN compacted in the IF space.

Accounting for exclusion of IF water from PG affects FCD$_{\text{EF}}$ and $\pi_{\text{PG}}$ values, and how FCD contributes to cartilage properties. The use of EF water instead of total water leads to increased estimates of local FCD by as much as 30% in the samples analyzed here, which in turn leads to increased estimates of $\pi_{\text{PG}}$ over that due to the nonlinear nature of the FCD-$\pi_{\text{PG}}$ relationship. This may clarify the role of $\pi_{\text{PG}}$ in the compressive aggregate modulus of cartilage. Although the slopes of the curves for $\pi_{\text{PG}}$, representing its contribution to modulus, were generally less than that for $\sigma_{\text{EF}}$ at 0–10% or 0–15% compression, they accounted for nearly all of $\sigma_{\text{EF}}$ by PG in bovine cartilage and young human cartilage, and nearly half of $\sigma_{\text{EF}}$ in old human cartilage at physiological salt concentrations. Previous studies using total water content attributed ~1/3 of the compressive modulus of cartilage to $\pi_{\text{PG}}$ (12,40). If the assumption of water partitioning were not true, the $\pi_{\text{PG}}$ amplification would be only that due to the nonlinear FCD-$\pi_{\text{PG}}$ relationship. With changes in COL content during growth, aging, and depth, the proportion of EF water available to interact with PG varies, resulting in changes in FCD$_{\text{EF}}$ and $\pi_{\text{PG}}$. This highlights the importance of interaction between the extracellular matrix components PG and CN, and the contribution from both components to FCD$_{\text{EF}}$ and $\pi_{\text{PG}}$.

Our results can be consistent with previous studies of cartilage compressive modulus at increased salt concentration (40,41). At the physiological salt concentrations considered here, nonelectrostatic contributions to $\pi_{\text{PG}}$, such as configurational entropy and mixing entropy, are likely to be small. Mixing entropy is generally considered to be very low at physiological PG concentrations (42), and configurational entropy has been suggested to be negligible at physiological salt concentrations using the Debye-Huckel model with a repulsive Lennard-Jones potential (13). However, at high salt concentrations that shield the electrostatic contribution, configurational entropy likely increases (12,13,42) and is thought to contribute to a larger proportion of the $\pi_{\text{PG}}$ and the compressive properties, with estimates of ~40–60% of compressive modulus values from experimental studies (12,40,41). Thus, the determination of electrostatic contribution at physiological condition from studies with increasing salt concentrations (40,41) is complicated by an increasing nonelectrostatic contribution to compressive properties. For the tissues analyzed here, with physiological concentrations of salt and PG, it appears that the electrostatic contribution from the charged GAGs is the major source of the $\pi_{\text{PG}}$.

The level of prestress exerted on the CN by $\pi_{\text{PG}}$ at zero strain appears to have an important impact on the compressive properties of the tissue, as suggested previously (43). The shift of the CN stress-strain curve from zero stress-strain state (i.e., 0, 0) into a prestress in tension at overall tissue zero strain indicates that the CN participates in compression, where the prestress is relieved. The compressive strain where the combined effect from high-sloped tension from the CN and low $\pi_{\text{PG}}$ from lower FCD$_{\text{EF}}$ likely contributes to the compressive softening previously observed at low strains (25,43,44). In addition, the degree of compression needed to relieve the CN prestress varies with growth, aging, and depth of the tissue. This appears to be related to the maturity of the CN (e.g., the presence of cross-links and COL orientation), because CN in immature cartilage provides much less restraining force than more-mature tissues, resulting in lower FCD$_{\text{EF}}$ with compression and weaker compressive properties. The contribution to compressive properties from both PG and CN, especially at low strains, gives articular cartilage its unique biomechanical properties.

Variations in FCD$_{\text{EF}}$ and $\pi_{\text{PG}}$ with the depth of adult cartilage appear to affect the overall functional properties of the tissue. The evening out of FCD$_{\text{EF}}$, $\pi_{\text{PG}}$, and $\sigma_{\text{CN}}$ profiles through the depth in young cartilage at 10% and 20% compression reflects the state of articular cartilage during steady-state loading. The changes in $\sigma_{\text{CN}}$ of normal young human cartilage from tension at zero strain to compression at lower applied compression (10% and 20%), and to tension in superficial CN while in compression in the deep layer CN at a high applied compression level (30%) are supported by previous studies of the CN under compression. The COL fibrils in superficial and middle layers may dissipate the strain under lower load, whereas COL fibrils in deep layers initially buckle or crimp and then distribute the load to the superficial layer under high load, leading to tension of the superficial COL fibrils and compression of deeper-zone COL fibrils (45). These results also have implications for cartilage function during dynamic loading. FCD is also inversely related to the hydraulic permeability of cartilage (3,4), and the dynamic stiffness and pressurization of cartilage depend on both the equilibrium compressive modulus and hydraulic permeability (46,47). Thus, our analysis of the FCD and $\pi_{\text{PG}}$ of cartilage provides insight into the function of cartilage during physiological loading.

The FCD$_{\text{EF}}$, $\pi_{\text{PG}}$, and $\sigma_{\text{CN}}$ profiles for old cartilage generally were similar to the trends observed for young cartilage at a normalized depth of ≥ 0.2, consistent with a dysfunction of the superficial layer with normal aging (6). With aging in old human cartilage, the highest levels of strains were observed in the middle layers and were not just localized
to the superficial layers as in young cartilage, resulting in high local FCD_{EF} and \( \pi_{PG} \) in the middle layers. The FCD_{EF} and \( \pi_{PG} \) peak in aged cartilage may indicate an abnormal distribution of stress through the depth of the tissue that may be unfavorable to the health of the matrix and chondrocytes in those regions. Although it is unclear whether this peak in \( \pi_{PG} \) in deeper layers is a result or cause of matrix degradation, these depth-varying compressive properties likely have important implications for the mechanobiology of cartilage and provide insight into the age-related changes that may lead to tissue degeneration.

The application of the FCD_{EF}-\( \pi_{PG} \) relationship to experimental biochemical data has the potential to predict \( \pi_{PG} \) for native cartilage of various sources (including human); depths; and states of growth, aging, and degeneration; as well as for engineered cartilaginous tissues with varying PG and COL contents. This may provide helpful insights into the design of (and possible targets for) tissue-engineered constructs on a macroscale with more uniform matrix composition (48) or on a microscale, at the level of individual cells, with more local, radially varying matrix regions (i.e., pericellular matrix) (49). Because only biochemical and compressive strain data are needed to estimate \( \pi_{PG} \), the calculations described above may provide a useful tool for elucidating, predicting, and targeting the biomechanical properties of native and engineered cartilaginous tissues, and relating the composition of a tissue to its function.

**SUPPORTING MATERIAL**

Materials and methods; methods, results, and discussion for bovine cartilage data; results and discussion for human cartilage data; and references, tables, and figures are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(II)0834-4.

The authors thank Dr. Amanda K. Williamson and Yehudit Falcozitz-Gerasso for providing raw data, and Barbara L. Schumacher for helpful discussions.

This study was supported by grants from the National Institutes of Health, the National Science Foundation, the Howard Hughes Medical Institute (to the University of California, San Diego, in support of R.L.S. through the HHMI Professors Program), and the Donald E. Beatty Center for Engineering Innovation (to S.M.K.). E.H.H. received a Graduate Research Fellowship from the National Science Foundation.

**REFERENCES**


