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An ionised/non-ionised dual porosity model of intervertebral disc tissue

Experimental quantification of parameters

J. M. Huyghe, G. B. Houben, M. R. Drost, C. C. van Donkelaar

Abstract The volume of the intrafibrillar water space – i.e. the water contained inside the collagen fibres – is a key parameter that is relevant to concepts of connective tissue structure and function. Confined compression and swelling experiments on annulus fibrosus samples are interpreted in terms of a dual porosity model that distinguishes between a non-ionised intrafibrillar porosity and an ionised extrafibrillar porosity. Both porosities intercommunicate and are saturated with a monovalent ionic solution, i.c. NaCl. The extrafibrillar fixed charge density of the samples is assessed using radiotracer techniques and the collagen content is evaluated by measurement of hydroxyproline concentration. The interpretation of the experimental data yields values for the intrafibrillar water content, the average activity coefficient of the ions, the Donnan osmotic coefficient, the fraction of intrafibrillar water, the stress-free deformation state, and an effective stress–strain relationship as a function of the radial position in the disc. A linear fit between the second Piola–Kirchhoff effective stress and Green–Lagrange strain yielded an effective stiffness: $H_e = 1.087 \pm 0.657$ MPa. The average fraction of intrafibrillar water was 1.16 g/g collagen. The results were sensitive to changes in the activity and osmotic coefficients and the fraction of intrafibrillar water. The fixed charge density increased with distance from the outer edge of the annulus, whereas the hydroxyproline decreased.

Introduction

Because of the role that the intervertebral disc may play in the etiology of low back pain, e.g. by disc herniations (Hickey and Hukins 1979; Osti et al. 1990; Chine and Luk 1995; Cooper et al. 1995) it is important to understand its mechanical function. The intervertebral disc consists of a gelatinous core – the nucleus pulposus – surrounded by a fibrous ring – the annulus fibrosus. The annulus fibrosus contains about 70% water by wet weight, 10–20% proteoglycans by dry weight, and 67% of collagen by dry weight (Eyre 1979). The annulus fibrosus is organized into circumferential fibrous lamellae that connect to the nucleus, end plates, vertebral bodies, and ligaments. There is a steady increase in the proportion of collagen and a decrease of proteoglycan content from the inner to the outer annulus (Eyre 1979; Lyons et al. 1981).

The annulus fibrosus has a strong swelling propensity because of the presence of the hydrophilic proteoglycans. The fixed negative charges on these proteoglycans are the main cause of tissue swelling. This swelling behaviour can be explained in terms of Donnan osmotic pressure (Urban and McMullin 1985) charge to charge repulsion forces (Eisenberg and Grodzinsky 1987), or both (Lai et al. 1991). The swelling tendency increases with the concentration of the fixed negative charges, which is called the fixed charge density. Urban and McMullin (1988), Maroudas and Bannon (1981), Katz et al. (1986), Maroudas et al. (1991) and Wachtel et al. (1995) have shown that part of the water contained

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J. M. Huyghe (✉), C. C. van Donkelaar
Engineering Mechanics Institute,
Eindhoven University of Technology, Eindhoven, The Netherlands
e-mail: j.m.r.huyghe@tue.nl

G. B. Houben, M. R. Drost
Department of Health Sciences, University of Maastricht,
Maastricht, The Netherlands

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in the tissue is absorbed by the collagen fibres. The proteoglycan molecules, because of their large size, are excluded from this intrafibrillar space. Hence, only the extrafibrillar water should be considered when computing the concentration of fixed charges inside the tissue. In other words, the effective fixed charge density is higher than it would be if computed from total tissue water content. Osmotic pressure is directly related to the fixed charge density and is therefore affected accordingly. The osmotic pressures in cartilage (Maroudas and Bannon 1981), and intervertebral disc (Urban and McMullin 1985) are quantitatively consistent with osmotic pressures determined from isolated proteoglycan solutions, provided that the osmotic pressures are computed from fixed charges per extrafibrillar volume. Maroudas et al. (1991) measured a dependence of the fraction of intrafibrillar water on the pressure, thereby demonstrating that the tissue is a dual porosity medium. The intrafibrillar water content was measured by low angle X-ray scattering. From the interaxial spacing of the collagen molecules, the mass of intrafibrillar water per collagen mass, φ_{ci}, was calculated. The intercollagen spacing and thus the intrafibrillar water content in cartilage is regulated primarily by the magnitude of the osmotic pressure gradient between the extra- and intrafibrillar compartments (Wachtel and Maroudas 1990; Maroudas et al. 1991). For the intervertebral disc, no studies have been performed to determine φ_{ci} under well-defined loading conditions. Urban and McMullin (1985) determined a value of 1.33 for φ_{ci} by comparing osmotic pressures of extracted proteoglycans with swelling pressures of disc slices, assuming that φ_{ci} was constant. However, having fitted their data with a straight line to obtain the value for φ_{ci}, they found that a quadratic relationship gave a better fit.

Furthermore, they had not matched the extracted proteoglycans and disc proteoglycans for glycosaminoglycan composition. Drost et al. (1995) performed transient confined swelling and compression experiments on cylindrical samples of canine annulus material. Compressive load is conferred to the sample by means of a loading piston that fits in the chamber. The sample is put in contact with a bathing solution of known osmotic strength by placing it on a filter through which the solution circulates (chemical load). The sample's height variation (piston displacement) is then measured over time as a function of mechanical and chemical load. Because of the well-defined sample geometry, and mechanical and chemical loads, the confined swelling and compression experiment is useful for determining constitutive properties of cartilaginous tissue. It will be used in the present study.

Inspired by the dual porosity insights of the above studies, Huyghe (1999) developed a finite deformation theory of cartilaginous tissues accounting for a non-ionised intrafibrillar porosity and an ionised extrafibrillar porosity, both saturated with a watery solution of a monovalent salt. Free communication exists between both compartments for the water, the cations and the anions. Diffusion and permeation within the intrafibrillar space is assumed negligible compared to the corresponding phenomena in the extrafibrillar space. This assumption is motivated by the much lower porosity of the intrafibrillar space compared to the extrafibrillar space.

The purpose of this study, therefore, is to quantify material parameters, that characterize the equilibrium dual porosity behaviour of canine annulus fibrosus. From confined swelling and compression experiments, the following chemomechanical properties of the annulus fibrosus were determined: the ions average activity coefficient, the Donnan osmotic coefficient, the fraction of intrafibrillar water, the stress-free deformation state, and an effective stress–strain relationship as a function of the radial position in the disc. To determine these parameters, dry weight, total fluid volume, collagen content and fixed charge density must be measured for each sample.

Theory

This section briefly reviews a set of differential equations that describe a cartilaginous tissue as a dual porosity medium in the isothermal and isotropic case (Huyghe 1999). We consider a porous solid ($\alpha = \mathrm{s}$) saturated by two liquid compartments, one intrafibrillar ($i = 1$) the other extrafibrillar ($i = 2$). The constituents of the liquid compartments are water ($\alpha = \mathrm{f}$), a monovalent cation ($\alpha = +$), and a monovalent anion ($\alpha = -$). A Greek character superscript denotes a constituent (solid, fluid, cation, anion) whereas a Roman subscript denotes a compartment (intrafibrillar, extrafibrillar). The Greek letter α refers to any constituent of the mixture, but the Greek letters β and γ refer to constituents of the liquid solution, i.e. water, cation or anion. We assume that the solid and all constituents β constituting the liquid compartments are intrinsically incompressible, i.e. if one adds a mass m of constituent α to the mixture, the added volume of the mixture is always proportional to the mass m, irrespective of the value m, the initial composition of the mixture or the state of deformation. We refer current descriptors of the mixture to an initial state of the porous solid. The deformation gradient tensor $\boldsymbol{F}$ maps an infinitesimal material line segment in the initial state of the solid onto the corresponding line segment in the current state. The relative volume change from the initial to the current state is the determinant of the deformation gradient tensor $J = \det\boldsymbol{F}$. If we introduce the

volume fraction N_i^β of constituent β in phase i per unit initial mixture volume, the mass balance equation is:

$$\frac{D^s N_i^\beta}{Dt} + \nabla_0 \cdot \left[N_i^\beta \mathbf{V}_i^\beta \right] = \hat{V}_i^\beta, \quad \beta = \mathrm{f}, +, - \tag{1}$$

if we exclude chemical reactions between the constituents and include mass transfer between same constituents of different phases. The superscript s means solid, the subscript i specifies the compartment: $i = 1$ intrafibrillar and $i = 2$ extrafibrillar. In Eq. (1), $\frac{D^s}{Dt} = \frac{\partial}{\partial t} + \mathbf{v}^s \cdot \nabla$ is the time derivative for an observer moving with the solid. $\mathbf{V}_i^\alpha = F^{-1} \cdot \mathbf{v}_i^\alpha$ is the Lagrangian form of the the velocity $\mathbf{v}_i^\alpha$ of constituent α in phase i, $\hat{V}_i^\alpha$ the volume of constituent α added onto phase i from the other phase per unit time and per unit initial mixture volume. Conservation of mass of each constituent requires:

$$\sum_{i=1,2} \hat{V}_i^\alpha = 0, \quad \alpha = \mathrm{s}, \mathrm{f}, +, - \tag{2}$$

while for the solid we find $\hat{V}^s = 0$. Saturation requires:

$$N^s + \sum_{i=1,2} \sum_\alpha N_i^\alpha = J \tag{3}$$

or if ionic volumes are neglected:

$$N^s + N_1^f + N_2^f = N^s + N^f = J \tag{4}$$

in which N^f is the total fluid volume per unit initial mixture volume. The electrostatic interactions between ions and the charged solid are accounted for by means of two electroneutrality conditions, one for the intrafibrillar and one for the extrafibrillar space. The electroneutrality conditions require that

$$C_i^{fc} + \sum_\alpha z^\alpha \frac{N_i^\alpha}{\bar{V}^\alpha} = 0, \quad i = 1, 2 \tag{5}$$

in which z^a are the valences, C_i^{fc} the fixed charge density per unit initial mixture volume and $\bar{V}^\alpha$ are the partial molar volumes of the solvents and solutes. The constitutive equation for the effective stress σ^{eff} is:

$$\sigma^{eff} = \sigma + p\mathbf{I} = \frac{1}{J} F \cdot \frac{\partial W}{\partial \mathbf{E}} \cdot F^T \quad , \tag{6}$$

and for the electrochemical potentials μ_i^β:

$$\mu_i^\beta = \frac{\partial W}{\partial N_i^\beta} + p + \frac{z^\beta F \xi_i}{\bar{V}^\beta}, \quad i = 1, 2, \quad \beta = \mathrm{f}, +, - \tag{7}$$

in which p is the fluid pressure, ξ_i the electrical potential of phase i and F is Faraday's constant. The frictional equations take the form:

$$-N_2^\beta \nabla_0 \mu_2^\beta = \sum_\gamma B_{22}^{\beta\gamma} \mathbf{V}_2^\gamma \ , \tag{8}$$

$$\mu_2^\beta - \mu_1^\beta = \sum_\gamma b^{\beta\gamma} \hat{V}_1^\gamma \ , \tag{9}$$

$$\begin{bmatrix} B_{22}^{\beta\gamma} & 0 \\ 0 & b^{\beta\gamma} \end{bmatrix}, \quad \beta = \mathrm{f}, +, -, \quad \gamma = \mathrm{f}, +, - \tag{10}$$

is a positive definite frictional matrix. For simplicity, and because collagen makes up no more than 20% of the volume of the tissue, we assume vanishing velocities $\mathbf{V}_I^\beta = 0$ in the intrafibrillar phase. This

means that the intrafibrillar compartment serves as a capacitance for the fluid and the ions flowing extrafibrillarly. The dynamic boundary conditions are given by no-jump conditions across the boundary for the extrafibrillar (electro)chemical potential of the ions and fluid, and the momentum balance of the boundary. The corresponding kinematic boundary conditions are the extrafibrillar ion and fluid flux balance across the boundary and the no-jump condition for the solid displacement. For the sake of readability, equations are written from now on for the one-dimensional case. The form of the Helmholtz free energy function W is chosen on grounds of simplicity and in agreement with common practices in physical chemistry and elasticity (Katchalsky and Curran 1967). We choose the energy function

$$W = \sum_{i=1,2} \left[\mu_0^{\mathrm{f}} N_i^{\mathrm{f}} + \mu_0^{+} N_i^{+} + \mu_0^{-} N_i^{-} - RT\Phi_i \left(\frac{N_i^{+}}{\bar{V}_i^{+}} + \frac{N_i^{-}}{\bar{V}_i^{-}} \right) \ln N_i^{\mathrm{f}} \right.$$
$$+ RT \frac{N_i^{+}}{\bar{V}^{+}} \left(\ln \frac{\gamma_i^{+} N_i^{+}}{\bar{V}^{+}} - 1 \right) + RT \frac{N_i^{-}}{\bar{V}^{-}} \left(\ln \frac{\gamma_i^{-} N_i^{-}}{\bar{V}^{-}} - 1 \right)$$
$$\left. + \frac{1}{\mathrm{hyp_{ww}a}} (x \ln x - x) + \frac{H_{\mathrm{e}}}{2} E^2 \right] \tag{11}$$

in which μ_0^{β} are the reference potentials, Φ_i the intra- and extrafibrillar osmotic coefficients, R the universal gas constant, T the absolute temperature, γ_i^{+} and γ_i^{-} the activity coefficients of the ions, $\mathrm{hyp_{ww}}$ hydroxyproline weight per wet weight, H_{e} the aggregate modulus, E the Green–Lagrange strain and

$$x = \frac{\mathrm{b} N_1^{\mathrm{f}}}{\mathrm{hyp_{ww}}} - \mathrm{c} \tag{12}$$

in which a, b and c are constants specifying the water-binding ability of the collagen. The hydroxyproline content $\mathrm{hyp_{ww}}$ is a measure of the collagen content. For the sake of simplicity, we assume in the subsequent derivations that the osmotic coefficients Φ_i and the activity coefficients γ_i^{-} and γ_i^{+} do not depend on the composition of the tissue. The anisotropic elasticity of the annulus fibrosus tissue is accounted for by means of an aggregate modulus, because the experiments that we consider force a sample to one-dimensional deformation. This paper deals with equilibrium states only. In this case, when a sample is in equilibrium with an external salt solution of concentration c_{ext}, the velocities of all components vanish and Eqs. (8) and (9) require that the electrochemical potentials

$$\mu_2^{\beta} = \mu_1^{\beta} = \mu_{\mathrm{ext}}^{\beta}, \quad \beta = \mathrm{f}, +, - \tag{13}$$

be uniform over the sample. In particular, the intrafibrillar fluid chemical potential μ_1^{f} equals, by substituting the energy function (11) into Eq. (7),

$$\mu_1^{\mathrm{f}} = p + \frac{\partial W}{\partial N_1^{\mathrm{f}}} = p + \mu_0^{\mathrm{f}} + \psi - \pi_1 \ . \tag{14}$$

The matric potential (Nitao and Bear 1996)

$$\psi = \frac{\mathrm{b}}{\mathrm{a}} \ln x \tag{15}$$

accounts for adsorption by the collagen, and the osmotic pressure

$$\pi_1 = RT\Phi \left(\frac{N_1^{+}}{N_1^{\mathrm{f}} \bar{V}^{+}} + \frac{N_1^{-}}{N_1^{\mathrm{f}} \bar{V}^{-}} \right) = RT\Phi_1 \left(c_1^{+} + c_1^{-} \right) \tag{16}$$

for osmosis by the ions; c_1^{+} and c_1^{-} are the intrafibrillar ionic concentrations. The matrix potential (Eq. 15) depends on the intrafibrillar fluid content N_1^{f} and the hydroxyproline content $\mathrm{hyp_{ww}}$. It represents the change in Helmholtz free energy of a volume of intrafibrillar water – initially equal to a unit volume – due to the vicinity of collagen. The osmotic pressure only depends on the ion concentrations c_1^{+} and c_1^{-}. The matrix potential is negligible in the extrafibrillar compartment because of the high fluid volume fraction:

$$\mu_2^{\mathrm{f}} = p + \mu_0^{\mathrm{f}} - \pi_2 \tag{17}$$

where π_2 equals, in agreement with Eqs. (11) and (7):

$$\pi_2 = RT\Phi_2\left(c_2^+ + c_2^-\right) \ . \tag{18}$$

Substituting Eqs. (14) and (17) into the Eq. (13), shows that the matric potential equals the osmotic pressure drop across the intra–extrafibrillar interface:

$$-\pi_2 = \psi - \pi_1 \ . \tag{19}$$

The water absorbing force of the collagen is balanced by the difference in osmotic pressure. Maroudas et al. (1991) performed experiments to determine a constitutive expression for the intrafibrillar matric potential ψ of articular cartilage. We fit (least squares) their relationship derived from X-ray data:

$$\mathrm{a} = 0.147 \times 10^{-9}\,\mathrm{m^4\,s^2\,kg^{-2}}, \quad b = 2.230 \times 10^{-3}\,\mathrm{m^3\,kg^{-1}}, \quad \mathrm{c} = 1.834 \ . \tag{20}$$

We assume that intervertebral disc is similar to articular cartilage, thus allowing us to use the same parameter values (Eq. 20). Substituting the energy function W (Eq. 11) into Eq. (7) for the ions, yields an expression for the electrochemical potentials of the ions:

$$\mu_i^\beta = \mu_{i0}^\beta + RT\ln\frac{\gamma_i^\beta N_i^\beta}{\left(N_i^{\mathrm{f}}\right)^{\Phi i}} + p + \frac{z^\beta F\xi_i}{\bar{V}^\beta}, \quad i = 1,2, \quad \beta = +,- \ , \tag{21}$$

which is consistent with classical expressions of electrochemical potentials (Katchalsky and Curran 1967). From Eq. (13), we know that in equilibrium the intra and extrafibrillar electrochemical potentials are equal. Adding this equality for the cations and anions, results in:

$$\left(\gamma_1^\pm\right)^2 c_1^+ c_1^- = \left(\gamma_2^\bullet\right)^2 c_2^+ c_2^- = \left(\gamma_{\mathrm{ext}}^\pm\right)^2 (c_{\mathrm{ext}})^2 \quad , \tag{22}$$

in which $\gamma_i^\pm = \sqrt{\gamma_i^+\gamma_i^-}$ are the average activity coefficients of the intrafibrillar, extrafibrillar and external solution. The electroneutrality conditions (5) require that

$$c_1^+ = c_1^- \ , \tag{23}$$

$$c_2^+ + \frac{C_2^{\mathrm{fc}}}{N_2^{\mathrm{f}}} = c_2^- \ . \tag{24}$$

Joining Eqs. (22–24) results in the Donnan equilibrium expressions:

$$2c_2^- = -c_2^{\mathrm{fc}} + \sqrt{\left(c_2^{\mathrm{fc}}\right)^2 + 4\left(\frac{\gamma_1^\pm}{\gamma_2^\pm}\right)^2 (c_1^+)^2} \ , \tag{25}$$

in which $c_2^{\mathrm{fc}} = C_2^{\mathrm{fc}}/N_2^{\mathrm{f}}$ is the fixed charge density per unit extrafibrillar volume. According to Eq. (13), Donnan equilibrium also holds between the extrafibrillar compartment and the external salt solution:

$$2c_2^- = -c_2^{\mathrm{fc}} + \sqrt{\left(c_2^{\mathrm{fc}}\right)^2 + 4\left(\frac{\gamma_{\mathrm{ext}}^\pm}{\gamma_2^\pm}\right)^2 c_{\mathrm{ext}}^2} \ , \tag{26}$$

and the osmotic pressure difference is obtained by equating the fluid chemical potentials of external solution and extrafibrillar compartment:

$$-\pi_{\mathrm{ext}} = p - \pi_2 \ . \tag{27}$$

For reasons of simplicity, and because fixed charges are not present in the intrafibrillar space or in the external solution, we assume the intrafibrillar activity coefficients and osmotic coefficients equal those in the external solution:

$$\gamma_1^{\pm} = \gamma_{ext}^{\pm}\Phi_1 = \Phi_{ext} \quad . \tag{28}$$

Under these assumptions, one can demonstrate the equivalence of mechanical pressures and osmotic pressure

$$-\psi = p = \pi_2 - \pi_1 = \pi_2 = \pi_{ext} \tag{29}$$

from Eqs. (19) and (25–27). This result is experimentally confirmed by Maroudas et al. (1991).

Methods

Sample preparation

The lower lumbar spines of three German shepherds (two males, one female) were harvested post-mortem. Body weights were 25, 28, and 40 kg and ages at death ranged from 1 to 3 years. The spines were dissected within 1–3 h after death. Spines were sawed off above the lumbar region, and below L7-S1. The ventral sides of the discs were partially freed of muscle tissue and of the longitudinal anterior ligament, leaving the surface of the discs intact for measurements. After dissection, the spines were sealed in plastic bags and kept frozen at −65 °C. Gleizes et al. (1998) found that freezing does not alter the biomechanical properties of intervertebral discs. Within two weeks, the frozen spines were sawed, excising in the radial direction parts of the discs in more or less rectangular slabs, of dimensions of roughly 15(radial) × 8(circumferential) × 8(axial) mm^3. The slabs were from discs L7-S1 to L2-3, and from either of the ventral (V), ventrolateral (VL), or dorsal (D) regions (Houben et al. 1997). The length axes of the slabs were approximately perpendicular to the outer surface of the annulus. Directly after the slabs were sawed, they were glued into Perspex (Stout Perspex and Plastic Industries, Rotterdam, The Netherlands) cylindrical holders having an i.d. of either 10.5 mm or 11.7 mm, with an o.d. of 15.0 mm, height 25–30 mm and a closed bottom. The slabs were glued with the outer surface of the annulus perpendicular to the axis of the holders, the surface sticking out about 1 mm. The glue was Tissue-tek (OCT Compound 4583, Miles Diagnostics, Elkhart, Ind. 46515, USA). During gluing, the slabs were kept frozen using liquid nitrogen (−196 °C). Upon contact with the liquid nitrogen, the Tissue-tek solidified. In case the outer edge of the annulus was polluted with blood, the surface was scraped clean with a scalpel, taking care not to damage it. Remnants of the spinal cord of dorsal specimens that had not been broken off cleanly during sawing were also removed with a scalpel. Within 4 days, the glued slabs were turned on a lathe. Radial samples of approximately 1 mm height with a 3.9 mm diameter were turned keeping the chisel and slabs cooled with liquid nitrogen. When possible, two or even three neighbouring samples per slab, with an interspace (lost material from the thickness of the chisel) of 1.0 mm, were produced. The outer samples had either an intact outer annulus surfaces or these were flattened on the lathe. Loss of material for flattening the outside surface of outer samples amounted to approximately 0.2–1.0 mm. The inner samples were flattened on both sides on the lathe. The approximate distance (frozen state) of the middle to the outer annulus edge was calculated of each sample, making it possible to infer quantities such as permeability, fixed charge density and porosity as a function of distance to the annulus edge. This distance was biased by measurement errors that in some cases could amount to 0.5 mm. The samples were put in aluminium cups and stored to a maximum of 21 days before being used in the experiment. During all the stages of preparation the samples were kept frozen using liquid nitrogen as a coolant.

Confined swelling and compression setup

Three identical experimental setups were used in parallel. A description of the testing apparatus is given elsewhere (Drost et al. 1995). The heart of the experimental setup for the confined swelling and compression measurements consisted of a cylindrical stainless steel chamber of diameter 4.030 ± 0.004 mm. The bottom of this chamber was formed by a sintered glass filter (pore sizes 16–40 μm, hydraulic permeability 10^{-12} m^4/Ns) whereas the top of the chamber was closed off by a piston.

Mechanical loading of the samples was accomplished by a cantilever arm on which the loading piston was mounted. Weights were hung on this arm, the compressive load of which was transferred via the piston. Chemical loading was accomplished by flowing a bathing solution of NaCl through the filter. The loading arm was connected to a DC operated linear variable displacement transducer (LVDT, Schaevitz, Fairfield, N.Y., USA) interfaced by a Labmaster 12 bit AD converter (Scientific Solutions Inc., Solon, Ohio, USA) to an IBM-AT (IBM, White Plains, N.Y., USA). A vibrator was attached to the setup to overcome sticking of the sample and piston to the chamber wall. 0.5 s after

Table 1. The loading stages of the experimental protocol. For the chemical load the average ±SD for all the experiments (n = 23) is given. Per experiment more exact values were used

	Conditioning	Swelling	Compression	Control
Mechanical load (MPa)	0.078	0.078	0.194	0.078
Chemical load (mol/l)	0.469 ± 0.013	0.159 ± 0.007	0.159 ± 0.007	0.469 ± 0.013
Duration	5 or 6 h	5 or 6 h	5 or 6 h	5 or 6 h

each sample point, the setup was vibrated at 50 Hz for 2 s. The data acquisition sampling frequency was 0.125 Hz. To allow free movement of the piston the measurement chamber was placed on a film of silicon oil and the circumference of the piston greased with Vaseline (Unilever, Rotterdam, The Netherlands).

Experimental protocol

Using a scalpel, the frozen sample was freed of irregularities that resulted from the turning. The sample was then placed in the chamber and the piston placed on top. During thawing the sample automatically expanded against the wall, filter and piston. Air was expelled from the setup by use of a vacuum pump, which was used as the salt solution was circulated. After the three samples had been placed in the cylinders, and the salt solution was circulating, data acquisition was started. Sample height was measured by the LVDT as a function of time. The temperature was kept at 22 ± 1 °C. The load scheme consisted of four stages: conditioning, swelling, compression and control. The duration of each stage was chosen sufficiently long to reach equilibrium (no change in sample height in time). The mechanical and chemical loads for all stages are tabulated in Table 1.

The bathing solution was either (average of all experiments ±SD) 0.159(±0.007) M or 0.469(±0.013) M. Exact values per experiment were determined from concentration measurements. To maintain the pH at a sufficiently high constant level the solutions were buffered with 5 mM Tris, pH 7.9. Computer driven electromagnetic valves switched the circulation from one bathing solution to the other. The changes of weights were done using a PC driven step motor. The total testing time was either 20 h (innermost samples) or 24 h (outermost samples), divided in equal parts per phase. The longer equilibration times for the outermost samples motivates this difference in experiment times.

For a sample to be chosen for further determinations after the confined swelling and compression experiment it had to be free of bone and nucleus material. An experiment was successful if (1) the four phases were well equilibrated, i.e. the mean absolute height change within the last hour of a plateau amounted to no more than 0.4% of the height at the end of the plateau, and (2) the height at the end of the control phase differed less than 5% of the conditioning height. From visual inspection of the time–displacement curves, these demands were regarded acceptable. The average percentage height loss ±SD of the height from the end of the conditioning to the end of the control stage was 1.72 ± 1.17% of the end height of the conditioning stage (n = 23). The average height change of the last hour in percent of the end height of the plateau was calculated for all stages: conditioning: 0.123 ± 0.093%/h, swelling: 0.056 ± 0.066%/h, compression: 0.106 ± 0.079%/h, control: 0.049 ± 0.050%/h.

The above criteria resulted in 23 successful experiments from a total of 79. The 23 samples were distributed among the three dogs as follows: eight from dog 1 (male; 28 kg), two from dog 2 (female; 25 kg), and 13 from dog 3 (male; 40 kg).

Data analyses

Chemical properties

Wet weight and height (volume), dry weight, hydroxyproline content, and the fixed negative charges of all samples were measured. From these quantities, total water content and fraction, collagen content, intra- and extrafibrillar water content, and fixed charge density were determined. At the end of the confined swelling and compression experiment, the height of the piston was measured and the sample was taken out and blotted to remove adhering water. Directly after this, the wet (total) weight was measured. The sample was stored frozen (−65 °C) before it was freeze dried (24 h) for dry weight determination.

Total water content and fluid fraction at sample height h were determined as follows:

$$V^{\mathrm{t}}(h_0) = h_0 A, \quad m_{\mathrm{e}}^{\mathrm{f}} = m_{\mathrm{e}}^{\mathrm{t}} - m^{\mathrm{s}}, \quad V^{\mathrm{f}}(h_{\mathrm{c}}) = \frac{m_{\mathrm{e}}^{\mathrm{f}}}{p^{\mathrm{f}}}, \quad V^{\mathrm{f}}(h) = V^{\mathrm{f}}(h_{\mathrm{e}}) + (h - h_{\mathrm{e}})A,$$
$$N^{\mathrm{f}} = N^{\mathrm{f}}(h) = \frac{V^{f}(h)}{V^{\mathrm{t}}(h_0)}\,. \tag{30}$$

with $V^t(h_0)$ the total sample volume at initial height h_0, A the area of the sample, m_e^f the fluid mass at the end of the confined swelling and compression experiment, m_e^t the total or wet mass at the end of the experiment, m^s the solid or dry mass (after freeze drying), $V^f(h)$ the fluid volume at height h, h_e the sample height at the end of the experiment, ρ^f the density of the fluid and $N^f(h)$ the fluid fraction at height h.

We assume the sample to be homogenous in composition and strain at the time of the chemical analyses. When the initial fluid fraction N_0^f at the initial height h_0 is known, the current fluid fraction N^f at the current height h can be calculated from:

$$N^f = J - 1 + N_0^f \ , \tag{31}$$

in which

$$J = h/h_0 \tag{32}$$

is the determinant of the deformation gradient defined prior to Eq. (1). Because of the homogenous one-dimensional configuration it is equal to the elongation factor h/h_0. Eq. (31) is obtained by substracting the initial form of Eq. (4) from its current form. After the dry weight was measured, the sample was cut in two, and the dry weights of each half were measured. One half was used for hydroxyproline determination, the other half for fixed charge density measurement.

To determine the collagen content, the dry wt% hydroxyproline (hyp_{dw}) was measured using a colorimetric assay (Stegemann 1958). A factor of 7.55 was employed to convert hydroxyproline to collagen (Adams and Muir 1976; Venn and Maroudas 1977). Fixed charge density was measured with the tracer cation method (Maroudas and Thomas 1970; Urban and Maroudas 1979), using ^{22}Na (Dupont de Nemours, Mechelen, Belgium) as the tracer. The samples were equilibrated for at least 48 h in a 0.015 M NaCl, 25% 20,000 polyethyleneglycol (PEG) solution, spiked with 0.1 μCi/ml $^{22}NaCl$. After dry weight measurement, the samples were put in dialysis sacs (molecular weight cut-off 15,000; Spectrum, Laguna Hills, Calif., 92653 USA) to prevent PEG from penetrating into the tissue. Equilibrating solutions were sealed off from contact with air to prevent discharge of carboxyl groups. Donnan equilibrium at an external concentration as low as 0.015 M requires that the fixed charge density approximately equals the Na^+ concentration, measured from the counts of the ^{22}Na (Urban and Maroudas 1979). Fixed charge density on dry weight basis, C_{dw}^{fc}, was calculated as follows: (1) 1 g of the equilibrating solution contained 1.125×10^{-5} mol NaCl, yielding y counts/min with x counts/min from the tissue given by $\nu = x\ (1.125 \times 10^{-5}/y)$ mol Na^+; (2) The fixed charge density on dry weight basis is given by $C_{dw}^{fc} = \nu/(\text{dryweight})$.

Equilibria

Based on the equilibria and using the previously determined quantities, the intra- and extrafibrillar water content, osmotic coefficients and aggregate modulus can be computed from the confined swelling and compression experiment. The aggregate modulus H_e is defined – in view of Eq. (11) – as the ratio of the second Piola–Kirchhoff stress S over the Green–Lagrange strain E. During an experiment there were four equilibrium states: conditioning ("0"), swelling ("sw"), compression and control. For the first three equilibrium states we determined: percent (1) intra- and extrafibrillar water fraction, (2) the fixed charge density on extrafibrillar water basis, c_2^{fc}, and (3) the second Piola–Kirchhoff effective stress as a function of elongation factor.

Intra- and extrafibrillar water content

The extrafibrillar water volume, V_2^f, is the difference between the amount total water, V^f, and intrafibrillar water V_1^f

$$V_2^f = V^f - V_1^f \ . \tag{33}$$

The boundary condition for momentum is given by:

$$-F/A = \sigma^{eff} - p \tag{34}$$

The osmotic pressure difference $\pi_2 - \pi_1$ is given by Donnan equilibrium, from Eqs. (16), (18), (28) and (29):

$$\pi_2 - \pi_1 = \Phi_2 RT\left(2c_2^- + c_2^{fe}\right) - 2\Phi_{ext} RT c_{ext} \ , \tag{35}$$

In Eq. (35), we use the equality

$$c_{\text{ext}} = c_1^+ = c_1^- \quad . \tag{36}$$

Equation (36) is a consequence of the intrafibrillar and the external solutions being in Donnan equilibrium with the extrafibrillar solution.

N_1^{f} depends on the matric potential ψ (Eqs. (12) and (15)), which equals the osmotic pressure difference (Eq. 29), which in turn depends on the fixed charge density c_2^{fc} (Eq. 35). c_2^{fc} depends in turn on N_1^{f} because the extrafibrillar water content depends directly on N_1^{f} through Eq. (33). An iterative procedure is needed to calculate N_1^{f} and thus c_2^{fc}. Iteration with Eqs. (12), (15), (29), (35) and (33) converges to a N_1^{f} with matching $\bar{V}^{\alpha}$.

The effective second Piola–Kirchhoff stress; stress-free elongation factors

The effective second Piola–Kirchhoff stress $S\ \partial W/\partial E$ is the current effective stress relative to some reference state. In most cases, a stress-free state is chosen as reference. As the stress-free state is not known a priori, a non-stress-free state, i.e. the end of the conditioning phase is chosen as the reference. Quantities pertaining to the reference are marked with a "0" suffix. In a one-dimensional situation, the effective second Piola–Kirchhoff stress S is related to the current effective Cauchy stress σ^{eff} according to Eq. (6):

$$S = \frac{1}{J}\sigma^{\text{eff}} \quad . \tag{37}$$

To determine σ^{eff} in the equilibrium states, one needs to calculate the osmotic pressure difference $\pi_2 - \pi_1$ between the extrafibrillar and external solutions. In equilibrium, the externally applied mechanical load F/A is balanced by the effective Cauchy stress σ^{eff} and the fluid pressure p (Eq. 34). The external load equals, using Eqs. (27), (36) and (37):

$$-F/A = \sigma^{\text{eff}} - p = \sigma^{\text{eff}} - (\pi_2 - \pi_{\text{ext}}) = JS - (\pi_2 - \pi_1) \quad . \tag{38}$$

$\pi_2 - \pi_1$ is derived from the anionic extrafibrillar concentration c_2^- and the osmotic coefficients using Eq. (35). The concentration c_2^- is computed from Eq. (26), which requires the activity coefficients. The mean activity coefficients depend on the ionic concentrations. For free solutions (salt and water), the mean activity coefficient is tabulated (Robinson and Stokes 1968). For 0.15 M NaCl, $\gamma_{\text{ext}}^{\pm}$ equals 0.755 and for 0.46 M NaCl it is 0.685. When the ionic concentrations inside can be determined, e.g. via partition studies, the mean activity coefficient inside can be inferred from (Eq. 22). Maroudas (1979) found that mean activity coefficients in cartilage lie mostly between 0.65 and 0.72 (external solution 0.15 M), decreasing when fixed charge density (on total fluid volume) increases. When the mean internal activity coefficient is not determined experimentally, it can be estimated from semi-empirical equations. The most complete treatment is given by Manning (1969) for low external concentrations. For higher concentrations, including physiologic, Kwak (1973) and Wells (1973) suggested modifications. According to these modifications, the mean activity coefficient is the product of a poly-ion/mobile ion interaction in the absence of salt, γ^{PM}, and mobile ion–mobile ion interaction, γ^{MM}:

$$\gamma_2^{\pm} = \gamma^{\text{PM}}\gamma^{\text{MM}} \quad . \tag{39}$$

Manning (1969) related the γ^{PM} to the ratio X of the fixed charge density to the concentration of free electrolyte (i.e. at the concentration of the co-ion) in the tissue:

$$\ln \gamma^{\text{PM}} = -0.5\xi \frac{X}{X + 2'} \tag{40}$$

where the factor ξ has the value 0.99 in accordance with the composition of disc proteoglycans (Urban et al. 1979). γ^{MM} is defined as the mean activity coefficient of the salt corresponding to the concentration of the co-ion in the polyelectrolyte solution (Wells 1973), or at the mean ionic strength of the polyelectrolyte solution (Kwak 1973; Freeman and Maroudas 1975).

We estimated the mean activity coefficient and c_2^- for the extrafibrillar compartment in absence of determined values of c_2^- and c_2^+ as follows. We used Eqs. (25)–(40) for an iterative procedure to estimate $\gamma_2^{\pm}$ and c_2^-. γ^{MM} was calculated using the least squares fitted graph data from Maroudas (1979) at the total mean ionic strength (i.e. $0.5 \times (c_{\text{tot}}^- + c_{\text{tot}}^+)$) of all the ions (intra- plus extrafibrillar) in the total

fluid volume, where the subscript "tot" refers to all the ions in the total fluid volume. γ^{PM} was calculated substituting for X the ratio of c_2^{fc} to the concentration of the co-ion (c_2^-). Because the proteoglycans are restricted to the extrafibrillar space we used a ratio based on extrafibrillar values here.

The initial value of $\gamma_2^{\pm}$ for the iterations was 0.70. This iteration loop was placed inside the iteration loop for the determination of N_1^f and c_2^{fc}.

The osmotic coefficient in free NaCl solution is a function of the NaCl concentration (Maroudas 1979), decreasing with increasing concentration. For concentrations of 0.15 M to 0.50 M it is almost constant at 0.924. The internal osmotic coefficient for cartilaginous tissues is split in two independent components, viz.:

$$\Phi_2 = \Phi^{PM}\Phi^{MM} \quad , \tag{41}$$

where Φ^{MM} is the osmotic coefficient in aqueous solutions, which is taken either at the co-ion concentration (Wells 1973) or at the mean ionic strength (Kwak 1973; Freeman and Maroudas 1975). Φ^{PM} represents the interactions between the poly-ions and mobile ions. Its dependence on γ^{PM} is defined by Manning (1969) as:

$$\Phi^{PM} = 1 + \ln \gamma^{PM} \quad . \tag{42}$$

The Φ^{MM} was calculated from a least squares fit of an exponential/polynomial function of the graph data from Maroudas (1979), substituting the total mean ionic strength on total fluid volume basis, analogous to the calculation of γ^{MM}. Using the formula:

$$\pi_2 = \Phi_{tot} RT\left(c_{tot}^- + c_{tot}^+\right) \quad , \tag{43}$$

one obtains an estimate of the osmotic coefficient F_{tot} for the total internal fluid volume.

Once the values of Φ_{ext} and Φ_2 have been determined, the effective second Piola–Kirchhoff stress S can be determined from Eqs. (35) and (38).

To derive the S as a function of the Green-Lagrange solid strain E, the three data points per experiment were fitted via a linear relationship between S and E:

$$E = \frac{1}{2}\left(\frac{h^2}{h_{sf}^2} - 1\right), \quad S = H_e\, E\,, \tag{44}$$

where h is the sample's height, h_{sf} is the height of the sample in the stress-free state of the solid, and H_e is the sample's effective linear stiffness.

In vivo values of parameters

At this point, we are in a position to quantify state variables of the sample at any equilibrium state. In particular, we are interested in the in vivo state. As the canine spines, from which the samples originate, were frozen shortly after death, we used the sample height measurement at the beginning of the experiment as the in vivo height. Data collection was started within 2 min after the initiation of thawing. The sample height at the beginning of the experiment is thus an approximate measure for the in vivo height, h_v, of the sample. The parameter values, based on h_v, are indicated with a subscript "v". We determined in vivo approximations of the fluid fraction, N_v^f, the stress-free elongation factor, $J_{sf,v} = h_{sf}/h_v$, the wet wt% hydroxyproline, $hyp_{ww,v}$, the fixed charge density on wet weight basis, $c_{ww,v}^{fc}$, and on total fluid volume basis, c_v^{fc}.

Results

Figure 1 shows the curves of the sample height versus time for two experiments. Panel a shows the result of an edge sample (distance from mid sample to annulus edge 0.27 mm), panel b of an inner sample (distance 2.42 mm). Comparing Fig. 1a and b, we see that the edge sample has a descending conditioning phase, but the inner sample an ascending conditioning phase. The compression phase in Fig. 1a shows, relative to the conditioning equilibrium height, more height loss for the edge sample. If we define edge samples as those with distance from mid sample to annulus edge ≤0.6 mm, and inner samples with distances larger than that, we have four edge samples and 19 inner samples. Three of those four edge samples have a descending conditioning phase, one of the 19 inner samples has a descending conditioning phase.

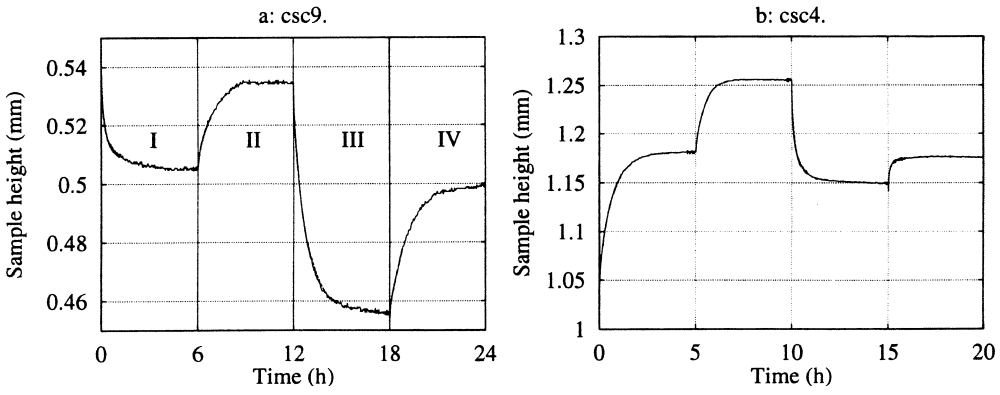

Fig. 1a, b. Sample height vs. time for two experiments: **a** edge sample, **b** inner sample. In **a** the four stages of the confined swelling and compression experiment are indicated: I = conditioning, II = swelling, III = compression, IV = control

Parameters as a function of distance to the annulus edge

We determined the fixed charge density on dry weight basis, c_{dw}^{fc} (Fig. 2a), the stress-free elongation factor relative to the conditioning equilibrium, $J_{sf,0} = h_{sf}/h_0$ (Fig. 2b), and relative to the swelling equilibrium, $J_{sf,sw} = h_{sf}/h_{sw}$, the dry wt% of hydroxyproline, hyp_{dw} (Fig. 2c). Assuming that the sample height within 2 min after the start of the experiment represents the in vivo thickness of the sample, we also calculated an estimation of the in vivo values of the following parameters: the fluid fraction, N_v^f, the stress-free elongation factor $J_{sf,v}$, and the wet wt% hydroxyproline, and fixed charge density on wet weight and fluid volume basis (hyp_{ww}, c_{ww}^{fc}, and c_v^{fc} respectively).

For the stress-free elongation factors $J_{sf,0}$, $J_{sf,sw}$ and $J_{sf,v}$, three of the 23 experiments were excluded from the analysis. These yielded effective stress-elongation factor results with a decreasing strain for increasing stress.

c_{dw}^{fc} rises with distance from the annulus edge; $J_{sf,0}$ and dry wt% hydroxyproline decrease with distance. The c_{ww}^{fc}, and c_v^{fc} (not plotted) also increased with distance from the annulus edge. We fitted (least squares) the plotted quantities of Fig. 4 with linear functions, and plotted these functions on the graph. The coefficients and statistics of the fits are given in Table 2.

For N_v^f, ρ^s, $J_{sf,sw}$, $J_{sf,v}$, and hyp_{ww}, a linear least squares fit gave no correlation with distance ($p_a > 0.05$). Extrema and average values ±SD of relevant parameters are listed in Table 3.

The mean activity and osmotic coefficients

The extrafibrillar mean activity and osmotic coefficients are listed in Table 4. Whereas the activity coefficients are reasonably constant over the stages, there is quite a large variation in osmotic coefficient. From conditioning to compression Φ_2 shows a decrease from 0.801 to 0.663 (17%).

Intrafibrillar water fraction

The iteratively calculated values of N_1^f per phase resulted in the following values for the ratio mass intrafibrillar water over mass collagen $\phi_{ci} = N_1^f \rho^f / M_{coll}$ (mean ± SD; $n = 23$): (1) conditioning: φ_{ci} = 1.21 ± 0.05 g/g, (2) swelling: φ_{ci} = 1.16 ± 0.04 g/g, (3) compression: φ_{ci} = 1.11 ± 0.05 g/g. The mean value for all phases is ϕ_{ci} = 1.16 ± 0.06 ($n = 69$). The above results indicate that the collagen fibres bind most water in the conditioning phase (highest φ_{ci}) and least in the compression phase. We have plotted c_2^{fc} as a function of the elongation factor J relative to the conditioning state. In Fig. 3 there are three points per experiment, joined by two line segments. The elongation factor was calculated with reference to the conditioning equilibrium. At swelling equilibrium, $J > 1$, at compression equilibrium, $J < 1$.

Effective stress

The effective second Piola–Kirchhoff stress S was calculated for the three equilibrium states. In Fig. 4, S is given as a function of the Green–Lagrange strain.

We fitted (Fig. 4; dashed line) 20 experiments with a linear function going through the origin according to Eq. (44). The average ±SD values for the effective linear stiffness and the stress-free elongation factors are given in Table 3. There was an increase ($p < 0.05$) of H_e from outer to inner annulus. Linear regression yielded: $H_e = (0.160 \pm 0.053)d + 0.448 \pm 0.098$ MPa, where d is the

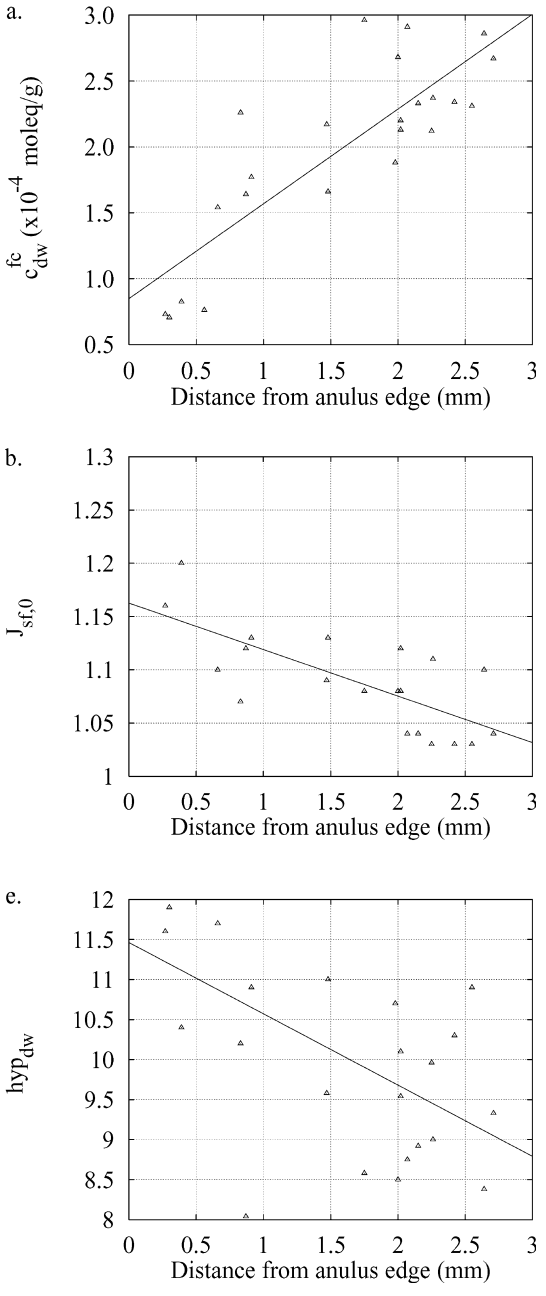

Fig. 2. Fixed charge density on dry weight basis c_{dw}^{fc} (**a**), stress-free elongation factor $J_{sf,0}$ (**b**), and wt% hydroxyproline (**c**) as a function of the distance of the centre of the sample to the annulus edge. For c_{dw}^{fc} and wt% hydroxyproline $n = 23$, for $J_{sf,0}$ $n = 20$. The plots have been fitted with linear least squares fits

distance from the annulus edge in mm; $R^2 = 0.33$, $p_a = 0.0076$. For the eight paired samples, the inner sample always had a higher H_e.

Discussion

This study introduces a protocol to determine mechanical properties of intervertebral disc annulus using a dual porosity electrochemomechanical theory. We divided the fluid compartment into intra- and extrafibrillar compartments based on the findings of Maroudas and colleagues (Maroudas and Bannon 1981; Katz et al. 1986; Maroudas et al. 1991; Wachtel et al. 1995) and Urban and McMullin (1985, 1988). While the experiment involves equilibrium states at very different values of osmotic pre-stressing, careful filtering of the osmotic pressures from the data yields effective stresses that are a monotonously increasing function of strain for 20 of the 23 samples. This result, consistent with thermodynamic restrictions, supports the dual porosity electrochemomechanic concept for annulus fibrosus tissue. The average linear stiffness between the effective second Piola–Kirchhoff stress and Green–Lagrange strain was found to be 1.087 ± 0.657 MPa. Best et al. (1994) found an aggregate modulus of 0.38 ± 0.16 MPa for human annulus fibrosus. Drost et al. (1995) found 0.66 ± 0.30 MPa for radial canine samples and 1.01 ± 0.31 MPa for axial samples. Houben et al. (1997) found

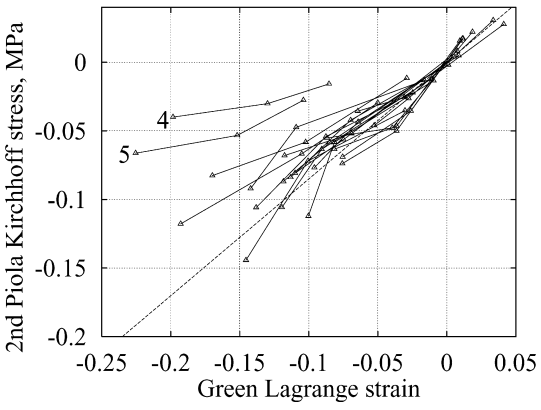

Fig. 3. The exfibrillar fixed charge density c_2^{fc} as a function of the elongation factor J relative to the conditioning height. The mass of intrafibrillar water per mass collagen φ_{ci} is iteratively calculated for each phase separately

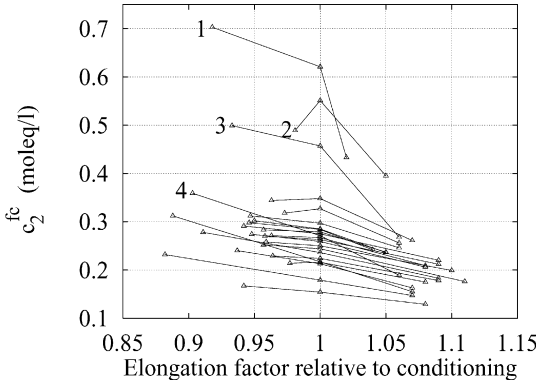

Fig. 4. The second Piola–Kirchhoff effective stress as a function of the Green–Lagrange strain ($n = 20$). The average linear fit (*dotted line*) is also plotted (slope = 1.09 MPa). Outliers have been labelled. Experiments 1–3 are left out because these yielded negative regression coefficients

Table 2. Linear least squares regression for parameters ($n = 23$ except 20 for $J_{s,f,0}$) as a function of distance of the mid-sample to the anulus edge: parameter = $a \times$ distance + b. In columns 2 and 3 the estimates of the coefficients a and b ± SD are given. Column 4 lists R^2, the coefficient of determination, and finally column 5 gives the two-tailed Student's t probability p_a, that the slope of the regression line is 0, i.e. that there is no relationship between parameter and distance

Parameter	a	b	R^2	p_a
c_{dw}^{fc}	$(7.2 \pm 1.0) \times 10^{-5}$ moleq g^{-1} mm^{-1}	$(8.5 \pm 1.8) \times 10^{-5}$ moleq g^{-1}	0.70	0.0000
$J_{sf,0}$	-0.044 ± 0.010 mm^{-1}	-1.163 ± 0.018	0.52	0.003
hyp_{dw}	$-0.89 \pm 0.28\%$ mm^{-1}	$11.46 \pm 0.50\%$	0.32	0.0047

Table 3. Average ± SD, and minimum and maximum of the parameter values ($n = 23$, except $n = 20$ for H_e, $J_{sf,0}$, $J_{sf,sw}$, and $J_{sf,v}$)

Parameter	Average ± SD	Minimum	Maximum
Distance (mm)	1.59 ± 0.79	0.27	2.71
c_{dw}^{fc} ($\times 10^{-4}$ moleq/g)	1.99 ± 0.68	0.706	2.960
c_{ww}^{fc} ($\times 10^{-4}$ moleq/g)	0.80 ± 0.28	0.283	1.170
c_v^{fc} (mol/l)	0.135 ± 0.047	0.045	0.202
hyp_{dw} (% of dry weight)	10.1 ± 1.2	8.04	12.80
hyp_{ww} (% of wet weight)	4.07 ± 0.80	2.64	6.94
H_e (MPa)	1.09 ± 0.66	0.208	2.550
$J_{sf,0}$	1.089 ± 0.046	1.030	1.200
$J_{sf,sw}$	1.013 ± 0.039	0.961	1.124
$J_{sf,v}$	1.194 ± 0.097	0.994	1.333
N_v^f	0.728 ± 0.068	0.531	0.847
Δh_{cc} (%)	1.7 ± 0.2	0.16	3.90

Table 4. Average ± SD of the extrafibrillar mean activity and osmotic coefficients

Phase	average $\gamma_2^{\pm}$ ± SD	Φ_2	n
Conditioning	0.623 ± 0.022	0.801 ± 0.033	23
Swelling	0.586 ± 0.025	0.710 ± 0.038	23
Compression	0.555 ± 0.024	0.663 ± 0.040	23
Total average	0.588 ± 0.037	0.725 ± 0.068	23

1.47 ± 0.41 MPa for canine radial samples. None of these values can be directly compared to our values, however, because they are all computed using a biphasic theory – not a dual porosity electrochemomechanical theory – and because of differences in experimental protocol. The stiffness computed using a biphasic theory is the sum of the elastic stiffness and the osmotic stiffness (Lai et al. 1991), using Eq. (6), (18) and (25):

$$\frac{\partial \sigma}{\partial J} = \frac{\partial \sigma^{\mathrm{eff}}}{\partial J} + \frac{\partial \pi_2}{\partial J} = H_{\mathrm{e}} + \frac{RT\Phi_2 c_2^{\mathrm{fc}}}{N_2^{\mathrm{f}}\sqrt{\left(c_2^{\mathrm{fc}}\right)^2 + 4\left(\frac{\gamma_{\mathrm{ext}}^{\pm}}{\gamma_2^{\pm}}\right)^2 c_{\mathrm{ext}}^2}}\left(1 - \frac{\partial N_1^{\mathrm{f}}}{\partial J}\right) \tag{45}$$

If we use c_2^{fc}, γ_{ext}, $\gamma_2^{\pm}$ and N_2^{f} corresponding to the conditioning phase in Eq. (45) and evaluate $\partial N_1^{\mathrm{f}}/\partial J$ from the change in φ_{ci} during the consolidation phase, we find a value of 1.83 MPa for the average biphasic stiffness. Direct fitting of the consolidation phase by means of biphasic theory results in a value of 1.77 ± 0.44 MPa. The samples at a distance from the edge < 1 mm ($n = 8$), resulted in a value of 1.54 ± 0.23 MPa, consistent with the value found by Houben et al. (1997) 1.56 ± 0.34 MPa ($n = 24$). The introduction of extra- and intrafibrillar compartments requires a new interpretation of ionic activity and osmotic coefficients. Determinations in the past of these quantities (Maroudas and Evans 1972; Maroudas 1975, 1979) have been based on Donnan osmotic theory for the total fluid compartment. This means that new experiments have to be designed to determine quantities such as γ^{MM}, γ^{PM}, Φ^{MM}, and Φ^{PM} for the extrafibrillar compartment. The laws that relate the above quantities to fixed charge density and ion concentrations (Eqs. 25–42)) are semi-empirical and the interpretation of these laws in terms of what concentrations should be substituted has been subject of discussion (Maroudas 1979). We used concentrations $\left(c_2^{+} + c_2^{-}\right)/2$ based on the total volume for the determination of γ^{MM} and Φ^{MM}, whilst for the poly-mobile ions interactions we chose concentrations on the basis of the extrafibrillar compartment. From the results of the coefficients per equilibrium stage we infer that the osmotic coefficient is especially sensitive to changes in the chemical and mechanical loads. These results indicate that for the determination of the osmotic coefficient it is important to accurately define the experimental conditions. It appears that the activity coefficients determined for the extrafibrillar compartment are smaller than the literature values for cartilage. For the mean activity coefficient for the ions in cartilage, Maroudas (1979) found the values to be in the range of 0.65–0.72 for an external solution of 0.15 M NaCl. For the extrafibrillar compartment, we found an average over the three equilibrium stages of 0.588 ± 0.037. After correction for the total volume, the value of 0.642 ± 0.026 was found which lies at low end of the range found by Maroudas for cartilage. The mean activity coefficient for the ions from our experiments is inferred from canine data in an experiment where mechanical loads were applied. For the determination of activity coefficients in cartilage, no mechanical load was applied in Maroudas' experiments (Maroudas and Evans 1972). For the osmotic coefficient we found 0.725 ± 0.068 and 0.868 ± 0.070. Maroudas (1975) inferred from cartilage data the relationship $\Phi_{\mathrm{cartilage}}/\Phi_{\mathrm{ext.\ solution}}2{\sim}0.8$. For an external Φ_{ext} of 0.942 (which is the value for molalities over 0.15 mol/kg) this would mean an internal osmotic coefficient of ~0.74, which lies closest to our extrafibrillar value. The two values should be compared with caution as our value relates to the extrafibrillar compartment of annulus fibrosus and Maroudas' value to the total fluid compartment of cartilage. The results of the stress–strain relationship proved to be sensitive to the ionic activity and osmotic coefficients, and the pressure–φ_{ci} relationship. It is therefore important that these quantities be determined accurately in future studies.

Fixed charge density, hydroxyproline and water content

To our knowledge, no data on fixed charge density of canine intervertebral disc were available prior to this study. All fixed charge density quantities $c_{\mathrm{dw}}^{\mathrm{fc}}$, $c_{\mathrm{ww}}^{\mathrm{fc}}$, $c_{\mathrm{v}}^{\mathrm{fc}}$ increased with distance to the annulus edge. For the fixed charge density on wet weight basis, we found an average of $(0.80 \pm 0.28) \times 10^{-4}$ mol eq/(g wet weight). Urban and Maroudas (1979) found for human intervertebral discs in the outer regions

of the annulus values between 0.7×10^{-4} and 1.3×10^{-4} mol eq/(g wet weight). They also found an increase of c_{ww}^{fc} going from the outer annulus inwards. For hydroxyproline and collagen we found relatively high values: hydroxyproline on dry weight basis 10.05 ± 1.24%. On wet weight basis the estimate of the hydroxyproline content in vivo was 4.07 ± 0.86%. For a conversion factor from hydroxyproline content to collagen content of 7.55, the collagen average on dry weight basis was: 75.9 ± 9.4%. Ghosh et al. (1976) examined collagen content in canine intervertebral disc as a function of age, spinal level and breed. They found no variation of collagen content with age or spinal level for the annulus fibrosus. For chondrodystrophoid and non-chondrodystrophoid breeds they found the annulus fibrosus collagen content to be around 55% (using a conversion factor of 7.4). Skaggs et al. (1994) found for human lumbar annulus fibrosus for the outer anterior annulus 62.6 ± 7.7% ($n = 9$), for the inner anterior annulus 59.3 ± 6.0 ($n = 8$), for the outer posterior annulus 63.0 ± 12.9% ($n = 9$), and for the inner posterior annulus 66.6 ± 5.9% ($n = 9$). They found no correlation with radial position for collagen. Best et al. (1994) calculated for human discs the hydroxyproline per wet weight. They reported a decrease of hydroxyproline per wet weight from outer to inner annulus, and lower values of hydroxyproline in the dorsal–lateral regions compared to the ventral region. The overall mean value on wet weight basis was 2.5 ± 1.0%. The increase of c_{ww}^{fc} with distance from the annulus edge is in accordance with the findings of Urban and Maroudas (1979). For the wt% hydroxyproline (wt% collagen) there is no uniformity in experimental studies. We found a decrease with distance to the annulus edge of hyp_{dw}, but no correlation for hyp_{ww}, whilst, albeit for human discs, Best et al. found a decrease of hyp_{ww}. Skaggs et al. (1994) found no correlation of hyp_{dw} with radial position. We did not find a radial dependence of water content N_v^f for the pooled results of all the samples. This might be due to the large scatter in the values of hydration per disc and disc region. Yet, of the five pairs of neighbouring samples (10 out of 23 samples), we found four with higher N_v^f of the inner sample, corroborating the generally accepted idea that water content of annulus fibrosus increases from outer to inner annulus (Urban and Maroudas 1979; Best et al. 1994; Skaggs et al. 1994). The results above indicate that it is best to compare hydroxyproline values of on a dry weight basis, because water content has been shown to have large fluctuations between discs and regions, so as to obscure variation as a function of radial position.

Effective stress–strain law and stress-free state of the sample

A linear relation between the radial stress and strain has been found, resulting in an average effective stiffness coefficient H_e of 1.09 ± 0.66 MPa ($n = 20$). In order to obtain a relationship between the stress and strain, the stress-free height of the sample, i.e. the height where the solid is stress free in the loading direction, had to be fitted as well. This height corresponds with zero strain. From Fig. 4 it can be seen that most Green–Lagrange strains were negative. This means that in most equilibrium states the solid was under compression. For the state of the sample at the beginning of the experiment, which is an approximation of the in vivo state, the stress-free elongation factor J_{sfv} was larger than unity for 19 out of 20 experiments (Table 3), meaning that the stress-free height of the sample was larger than the approximate in vivo height. Thus it seems that in vivo the solid is under compression in radial direction. A remarkable finding is that H_e increased from the outer annulus inwards. Also for the paired samples, the inner sample always had a larger H_e than the outer one. In tensile tests of annulus fibrosus it was found by Galante (1967) for annulus specimens in circumferential direction, and by Skaggs et al. (1994) for single lamellar annulus fibrosus specimens, that the stiffness decreased going from the outer annulus inwards. However, these results refer to tensile stiffness in the circumferential direction of the annulus, and not to compressive stiffness in the radial direction. Collecting more data points per experiment to fit a stress–strain curve is difficult because of the long equilibration times needed per point, and the deterioration (loss of proteoglycan and autolysis) of the sample in the course of the experiment.

The influence of the intrafibrillar water fraction N_1^f on calculated results

We calculated the N_1^f by using a curve fit of the pressure–N_1^f relationship based on articular cartilage data from Maroudas et al. (1991), because we had no pressure–N_1^f relationship for the annulus fibrosus. We found an average value of $\varphi_{ci} = 1.16 \pm 0.06$ ($n = 69$) for the three equilibrium states for φ_{ci}, which compares reasonably with the value of 1.33 determined by Urban and McMullin (1985). The in vivo water content was $N_v^f = 0.728 \pm 0.068$, from which $N_{1,v}^f = 0.240 \pm 0.017$ was intrafibrillar, indicating that the shielding of water by the collagen from the proteoglycans is significant in the canine annulus fibrosus. Even if a model neglecting the intrafibrillar water may reproduce some features of the swelling mechanics of the annulus fibrosus, using such model for the prediction of the electromechanical environment by the cells of the disc, may lead to major errors in Donnan pressures and potentials.

Conclusions

Confined swelling and compression experiments have been combined with a number of physico-chemical measurements to separate the elastic, osmotic, and viscous contributions of annulus fibrosus to the overall behaviour of the intervertebral disc. Across several equilibrium states involving different values of osmotic pre-stressing and mechanical load, careful filtering of the osmotic pressures from the data yields effective stresses that are a monotonously increasing function of strain for most samples. Although the experiment necessarily takes the samples away from their natural in vivo state, this study has reconstructed from the experimental data the in vivo value of a number of state parameters. The fixed charge density increased with distance from the outer annulus edge, hydroxyproline decreased. The difference between stress-free state and in vivo state of the elastic component of the disc is found to be significant and non-homogenously distributed across the annulus fibrosus. The existence of an initial homogenous stress-free state in the unloaded disc, as usually assumed in finite element analyses of the disc (Shirazi-Adl et al. 1984; Simon et al. 1985), should therefore be questioned.

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