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Nonlinear osmotic properties of the cell nucleus

John D. Finan^{*,#}, Kevin J. Chalut[#], Adam Wax[#], and Farshid Guilak^{*,#}

^{*} Department of Surgery, Duke University Medical Center, Durham, NC 27710

[#] Department of Biomedical Engineering, Duke University, Durham, NC 27708

Summary

In the absence of active volume regulation processes, cell volume is inversely proportional to osmolarity, as predicted by the Boyle Van't Hoff relation. In this study, we tested the hypothesis that nuclear volume has a similar relationship with extracellular osmolarity in articular chondrocytes, cells that are exposed to changes in the osmotic environment in vivo, and furthermore, we explored the mechanism of the relationships between osmolarity and nuclear size and shape. Nuclear size was quantified using two independent techniques, confocal laser scanning microscopy and angle-resolved low coherence interferometry. Nuclear volume was osmotically-sensitive but this relationship was not linear, showing a decline in the osmotic sensitivity in the hypo-osmotic range. Nuclear shape was also influenced by extracellular osmolarity, becoming smoother as the osmolarity decreased. The osmotically-induced changes in nuclear size paralleled the changes in nuclear shape, suggesting that shape and volume are interdependent. The osmotic sensitivity of shape and volume persisted after disruption of the actin cytoskeleton. Isolated nuclei contracted in response to physiologic changes in macromolecule concentration but not in response to physiologic changes in ion concentration, suggesting solute size has an important influence on the osmotic pressurization of the nucleus. This finding in turn implies that the diffusion barrier that causes osmotic effects is not a semi-permeable membrane, but rather due to size constraints that prevent large solute molecules from entering small spaces in the nucleus. As nuclear morphology has been associated previously with cell phenotype, these findings may provide new insight into the role of mechanical and osmotic signals in regulating cell physiology.

Keywords

cell mechanics; chromatin; cartilage; osteoarthritis

Introduction

The ability to perceive and respond to biophysical factors, such as mechanical or osmotic stresses, is critical for normal cell behavior. For example, many cell types exhibit biological responses to physical stimuli such as mechanical deformation, hydrostatic pressure, fluid shear stress, or osmotic stresses. These physical parameters are often coupled to one another in tissues that possess a charged, hydrated, extracellular matrix, such as articular cartilage^{40, 44, 48, 49, 52–54, 66, 72}. Such stresses may alter cell behavior by initiating traditional cell signaling processes involving the activation of ion channels^{32, 36, 46, 64} or other membrane-bound receptors^{17, 19}. It has also been hypothesized that physical stresses propagate through the cell and act directly on the nucleus and other intracellular organelles, exerting a biological influence at a subcellular level¹⁶. In this respect, the physical conformation of the nucleus is associated

Address correspondence to: Farshid Guilak, PhD, 375 MSRB, Box 3093, Duke University Medical Center, Durham, NC 27710., Tel. 919-684-2521, FAX: 919-681-8490, email: guilak@duke.edu.

with many important biological phenomena. For example, the nuclei of epithelial cells enlarge dramatically when they become cancerous⁵. Chromatin condensation is one of the first changes to occur in apoptosis⁴². Nuclear lamins A/C accumulate as stem cells differentiate^{7, 18, 62} and the stiffness of the lamina network increases as a result⁵⁸. Interestingly, lamin B1, the only sub-type that does not affect the stiffness of the lamina network⁴⁵, is unchanged during differentiation. The significance, if any, of these physical changes can be better interpreted through more detailed knowledge of the biophysical properties of the cell nucleus.

It is now apparent that extracellular matrix interacts with the cytoplasm and other intracellular organelles such as the nucleus via a solid-state connection to the cytoskeleton^{29, 41, 47, 74}. There has been considerable speculation that this connection provides a mechanism by which extracellular stresses can be transmitted intracellularly in a manner that can alter cell signaling directly at the level of the nucleus^{37, 47}. Osmotic stress, on the other hand, does not depend on solid connections, but rather is “transmitted” by the diffusion of ions and water through fluid compartments and is modulated by local fixed charge density. Since the cell membrane is permeable to water, osmotic stress is rapidly propagated to the interior of the cell in the presence or absence of solid structures in the cytoplasm. However, the physical effects of extracellular osmotic stress on the cell nucleus, as well as the intrinsic physicochemical properties of the nucleus, are not well understood.

A few previous studies have examined the physical and biomechanical properties of the cell nucleus. The nucleus is stiffer and more viscous than the cytoplasm^{23, 25, 35}, potentially contributing to the stiffness of the cell as a structure¹². Furthermore, defective nuclear mechanics due to genetic alterations in nuclear cytoskeletal proteins compromise the mechanical integrity of the whole cell⁸. The nuclear envelope is supported by a network of intermediate filaments called lamins and filled with chromatin³⁰. The stiffest part of the nucleus is the lamin network, while the most viscous part is the chromatin. Both appear to show power law dynamics after the application of a step load²⁰. Computational studies have shown that the mechanical response of the whole nucleus is highly sensitive to the stiffness of the nuclear lamina⁷³. Chromatin contains many charged groups that limit its compaction by repelling one another and cause it to condense or expand in response to changes in ion concentration^{67–69}. Small molecules and ions diffuse freely into and out of the nucleus through the nuclear pore complexes that populate the nuclear envelope⁵⁷. In charged hydrated tissues such as cartilage, bulk compression of the tissue changes the shape and volume of the chondrocyte and its nucleus in a coordinated manner^{10, 33, 43}. However, if the F-actin cytoskeleton is disrupted with cytochalasin D, the effects of tissue compression on nuclear shape disappear, but the effects on volume remain³³.

Osmotic loading affects a range of biological processes in articular chondrocytes including calcium signaling²⁶, protein synthesis⁶, gene expression³⁹ and cytoskeletal reorganization^{15, 27, 34}. The physical response that accompanies these biological responses is well-understood at the whole-cell level. When active responses are suppressed, the reciprocal of osmolarity exhibits a linear relationship with cell volume, as predicted by the Boyle Van't Hoff relation⁵⁵. Many mammalian cells obey the Boyle Van't Hoff relation, including chondrocytes^{11, 34}, neutrophils⁷⁰ and human islet cells²². Changes in the extracellular osmotic environment cause intracellular changes such as alterations in genome architecture¹ due to an increase of macromolecular crowding in the cytoplasm that causes histone aggregation through an excluded volume effect⁶¹. The volume of the chondrocyte nucleus changes with changes in osmolarity⁵⁶ but it is not known if the osmotic response of the nucleus shares the linear character of the cell response. We hypothesized that the nucleus, similar to the cell, would behave as an ideal osmometer, following the Boyle Van't Hoff relation. The goal of this study was to examine this hypothesis by systematically quantifying the dynamic and equilibrium morphologic response of the cell nucleus to changes in extracellular osmolarity using two

independent methods, confocal microscopy and angle-resolved low coherence interferometry (a/LCI). Further experiments examined the mechanisms of the observed behavior.

Results

Cell volume was found to be linearly related to inverse normalized osmolarity throughout the domain tested, indicating that chondrocytes obey the Boyle Van't Hoff relation for the volumetric response of an ideal osmometer. The osmotically active volume fraction of the chondrocyte was determined from a linear fit to be 67% ($R^2=0.97$).

Nucleus volume was also sensitive to osmolarity but in a non-linear manner. The nucleus volume increased linearly with inverse normalized osmolarity in the hyper-osmotic range. In the hypo-osmotic range, the slope of the relationship decreased continuously, suggesting an upper limit to nuclear expansion. The key features of the relationship between nucleus volume and osmolarity were found to be the same regardless of whether it was measured using fluorescent microscopy or a/LCI (Fig. 1A). For the sake of comparison, the Ponder's plot data was separated into four cases representing the two different structures, the cell and the nucleus (as measured by fluorescence microscopy), and two different ranges, hyper-osmotic and hypo-osmotic. In each case, the data was fit to a straight line to allow comparisons of the osmotic sensitivity to be made (Fig. 1B). A two factor ANOVA revealed a main effect of structure (cell vs. nucleus), no main effect of osmotic range and a significant interaction between structure and osmotic range. The osmotic sensitivity of the cell did not change significantly between the hyper- and hypo-osmotic ranges but the sensitivity of the nucleus did change. The nucleus was more sensitive than the cell in the hyper-osmotic range and less so in the hypo-osmotic range. The nucleus volume response in the hyper-osmotic range was highly linear ($R^2 = 0.992$) and the y-intercept of the linear fit was 0.204. This finding implies that the solid volume fraction of the nucleus is 20.4%.

An existing mathematical model describing osmotic swelling of gels² was modified to introduce a membrane representing the nuclear lamina (see Materials and Methods). This model was fit to the hypoosmotic volume data as measured by confocal microscopy to estimate the properties of the nuclear membrane. The optimal fit was obtained when the dilational modulus of the nuclear envelope was 0.023N/m and the nuclear envelope became taut at an extracellular osmolarity of 321 mOsm (see Fig. 2). The r^2 value of the fit was 0.9998. The model was optimized from a range of initial values to ensure the uniqueness of the reported optimal result. The optimization was repeated for several values of the K , the bulk modulus of the nucleus. It was found that the optimal value of the membrane stiffness scaled with the bulk modulus over four orders of magnitude from 100 Pa to 1 MPa. However, the optimal value of the osmolarity at which the nuclear membrane became taut remained the same across this range.

Extracellular osmolarity influenced nucleus shape (Fig. 3). The contour ratio increased linearly with inverse normalized osmolarity in the hyper-osmotic range, indicating that the shape was becoming smoother. The slope of the relationship decreased in the hypo-osmotic range, appearing to approach a limit. This behavior was similar to the behavior of the relationship between nucleus volume and inverse normalized osmolarity.

The relationship of shape and size to osmolarity was also apparent qualitatively (Fig. 4). Cell shape was approximately circular (i.e., spherical) at every osmolarity, except for small distortions at the peak value. The size of the cell increased continuously with decreasing osmolarity throughout the range shown. The irregular shape of the nucleus at elevated osmolarity became approximately circular and the nucleus expanded as the osmolarity decreased.

The transient changes in the cross-sectional area of the cell and nucleus changed monotonically with time, showing negligible evidence of volume recovery (Fig. 5), as evidenced by the fact that an exponential expression with a single time constant fit the data well. In the cell, the time constant for volume decrease in response to hyper-osmotic stress was lower than that for volume increase with hypo-osmotic stress. Other differences between time constants were not significant at 95% confidence (Table 2).

The rate of change in nucleus shape was lower than that of nucleus size. The transient of the contour ratio required 120 seconds to reach its peak value. As a result, the perimeter length of the nucleus initially increases rapidly under hypo-osmotic loading to a peak at about 15 seconds before decaying steadily for the rest of the experiment (Fig. 6) as the shape evolves to accommodate the constant cross sectional area within a shorter perimeter.

The morphological response of the nucleus to hyper-osmotic loading was not influenced by cytochalasin D treatment. Upon transition from 380 mOsm media to 580 mOsm media, nuclear volume decreased by approximately 27%, regardless of whether they had been pretreated with cytochalasin D (Fig. 7A). A two-way factorial ANOVA revealed a main effect of osmolarity but no main effect of cytochalasin D treatment and no interaction between osmolarity and cytochalasin D treatment. The reduction of volume at high osmolarity was highly significant ($p < 0.001$). Similarly, the contour ratio decreased by 5%, regardless of treatment. Two-way factorial ANOVA showed a main effect of osmolarity but no main effect of treatment and no interaction between treatment and osmolarity. The reduction in the contour ratio at high osmolarity was also highly significant ($p < 0.001$) (Fig. 7B).

Isolated nuclei contracted in response to increased macromolecule concentration but not in response to increased ion concentration. A highly significant ($p < 0.001$) reduction of 9% in the volume of isolated nuclei occurred when they were subjected to an increase in macromolecule concentration consistent with observed hyper-osmotic shrinkage of the cell. However, a similar increase in ion concentration caused no detectable change in the volume of the isolated nuclei (Fig. 8). The macromolecules used were 10 kDa dextrans, which are several orders of magnitude larger than ions but still small enough to pass through the pores in the nuclear envelope. This was confirmed with rhodamine-labeled dextrans (Fig. 9).

Discussion

The findings of our study indicate that the equilibrium osmotic response of chondrocytes matches that of an ideal osmometer, as described by the Boyle Van't Hoff relationship, and the transient results show negligible recovery over time. Therefore, the equilibrium and transient responses of the cells suggest that they behave in a passive manner under these experimental conditions. The transient changes in cross-sectional area of the nucleus also showed negligible recovery. However, under these same conditions, the equilibrium volumetric response of the nucleus to changes in osmolarity, as measured by two independent methods, was found to be highly non-linear. The mechanisms involved in this non-linear physicochemical behavior are not completely understood, but changes in the shape of the nucleus were found to parallel changes in the size of the nucleus under equilibrium conditions, suggesting that the two are interdependent. By contrast, shape change lagged size change in transient experiments. Further experiments suggest that the mechanism of nuclear morphology change is not cytoskeletal force transmission or concentration of intracellular ions but concentration of intracellular macromolecules.

In a previous study of nuclear size in chondrocytes subjected to a single step change of 200 mOsm, nuclei changed less than cells in the hypo-osmotic case but the changes were not significantly different in the hyper-osmotic case⁵⁶. These findings are in agreement with the

results of the present study, where we found that the slope of the Ponder's plot (i.e. the volume response) was only 0.24 for the nuclei while it was 0.66 for the cells in the hypo-osmotic range. However, in contrast to the previous results, we found a difference between the cell and nucleus in the hyper-osmotic range, where the slope of the Ponder's plot was 0.67 for the cells and 0.8 for the nuclei. The difference between the cell and nucleus responses was greater in the hypo-osmotic range than in the hyper-osmotic range (see Fig. 1B), supporting the asymmetry seen in the previous study⁵⁶.

The hyper-osmotic response of the nucleus on a Ponder's plot was found to be linear with a y-intercept of 20.4%, suggesting that this is the solid volume fraction of the nucleus. This agrees with the findings of Rowat et al., who reported that micropipette aspiration of nuclei reduced their volume but it was difficult to reduce the nuclear volume by more than 70%⁶³. Despite the linearity of the response, the nucleus does not have the semi-permeable membrane characteristic of an ideal osmometer. The nuclear envelope is punctuated by pores that render it permeable to small ions⁵⁷. There was a significant reduction in the volume of isolated nuclei loaded with increased concentrations of macromolecules but no significant change in the volume of isolated nuclei loaded with increased concentrations of ions (Fig. 8). This suggests that the nucleus dilates because the macromolecular concentration in the cytoplasm changes when the cell volume changes. These findings are consistent with other reports in the literature. Albiez et al. reported that nuclear architecture was altered by hyper-osmotic loading with saline but that the effect was abolished by permeabilization of the cell membrane¹. Richter et al. reported that changes in ion concentration did not influence the nuclei of permeabilized cells but changes in macromolecule concentration did⁶¹.

Macromolecules may exert an osmotic stress even on systems such as the nucleus that are not surrounded by a semi-permeable membrane. Size exclusion of large molecules from small tortuous spaces in a gel creates dilute regions that are osmotically pressurized. The size exclusion effect is quantified by the partition coefficient, the ratio between solute concentrations inside and outside the gel. If the partition coefficient is less than one, the gel is osmotically pressurized. Experimental evidence for this phenomenon in alginate beads along with a theoretical analysis was provided by Albro et al.². They found that the partition coefficient of a solute molecule is a function of its size, since larger molecules are excluded from a larger fraction of the pores in the gel. Monovalent ions are small enough to enter even the smallest pores so that no osmotic response is expected for osmotic loading with saline in this model. On the other hand, large molecules such as 10 kDa dextrans are expected to have a partition coefficient substantially less than unity and exert substantial osmotic pressure. This is indeed the case for isolated chondrocyte nuclei (Fig. 8). The increase in macromolecular concentration was intended to model the change in the cytoplasm of a cell subject to an extracellular osmolarity of 580 mOsm. However, the reduction in the volume of the isolated nuclei was only 9% whereas the reduction of volume for nuclei inside cells subjected to 580 mOsm was 28%. This may be because the solution used to load isolated nuclei contained only macromolecules of 10 kDa. The cytoplasm contains larger molecules that may have smaller partition coefficients with the nucleoplasm and hence exert a larger osmotic load. Also, the cytoplasm contains molecules too large to pass through the nuclear pores that exert a conventional osmotic pressure on the nucleus. Hence, it is unsurprising that the volume reduction in isolated nuclei is less than for nuclei in situ.

The model presented by Albro et al.² predicts a linear relationship between osmolarity and volumetric strain so the Ponder's plot of the model output has a continuously declining slope. However, the decline in the slope is not sharp enough to fit the abrupt plateau in the observed volume data in the present study. This characteristic, combined with the observed correlation between shape and volume, motivated a modification of the model to include a membrane tension term to model the mechanical integrity of the nuclear lamina (see Materials and

Methods). This model allowed estimation of the stiffness of the nuclear lamina along with the osmolarity at which the nuclear lamina becomes taut. The value determined for the stiffness of the nuclear lamina depends heavily on the value used for the bulk modulus of the nucleus, a property that can only be estimated from the available literature. A more accurate value for this property would increase the power of this approach to non-invasively determine the mechanical properties of the nuclear membrane. The osmolarity at which the nuclear lamina becomes taut, 321 mOsm, is not sensitive to the value of the bulk modulus. It is significant that this value lies below the osmolarity experienced by chondrocytes (380 mOsm), suggesting that physiologic changes in cartilage osmolarity (secondary to mechanical loading) would result in changes in nucleus size in situ.

Nucleus shape changed more slowly than the nucleus size under hypo-osmotic load (Fig. 6). The mechanism of this behavior is unknown but may result from inhomogeneity in the viscoelasticity of the nucleus. Previous studies suggest that the nucleoplasm creeps more rapidly than the lamina,²⁰ so it is possible that nuclear morphology is determined initially by the nucleoplasm but thereafter is increasingly influenced by the lamina as the stiffness of the nucleoplasm decays. Since the lamina acts mechanically as a membrane, its influence is to move the nucleus towards a smoother shape that minimizes surface area and the length of the perimeter of the cross-section. The changing geometry of the nucleus may reflect the redistribution of strain between these two structures according to their changing stiffnesses. Further work is necessary to fully understand this phenomenon.

The transient response of the cell cross-sectional area was significantly slower in the hypo-osmotic condition than in the hyper-osmotic condition (see Table 2). While an equivalent change in osmolarity was applied in both conditions (100 mOsm), it is important to note that the volume change predicted by the Boyle Van't Hoff relation is related to the reciprocal of osmolarity. In this case, the hypo-osmotic condition is a 35% increase in the reciprocal of osmolarity while the hyper-osmotic condition is a 21% decrease. The hypo-osmotic response may equilibrate more slowly because it represents a larger effective change. Also, artificial disruption of the F-actin cytoskeleton has been shown to slow volume change in both hypo- and hyper-osmotically loaded chondrocytes⁵⁶. Hypo-osmotic loading disrupts the F-actin cytoskeleton in untreated chondrocytes but hyper-osmotic loading does not^{27, 34}. Thus, cytoskeletal disruption and remodeling may also play a role in the observed difference in the time constants.

This study relies on measurement of volume inferred from the cross-sectional area of a cell or nucleus in a two dimensional image for the transient and equilibrium experiments on nuclei within intact cells. Therefore, an important assumption in the interpretation of this data is that the cell or nucleus in question is a sphere. In this study, chondrocytes are observed to be quite spherical, but nuclei are often spheroidal and may even have more complicated shapes as indicated in the discussion of evolving contour ratios. For this reason, measured values were normalized to the iso-osmotic condition rather than presented as absolute values. Exact knowledge of the three-dimensional shape is required to relate cross-sectional area to absolute volume but the relationship between area dilatation and volume dilatation is less sensitive. The ratio of the reference and deformed volume for an isotropic deformation field is equal to the ratio of the reference and deformed cross-sectional areas raised to the power of 1.5, regardless of geometry. Isotropic deformations were assumed for the cell and nucleus in these experiments. To validate this approach, three dimensional confocal images were taken of stained nuclei in intact chondrocytes at three different osmolarities and used to calculate volume. Then, the three dimensional images were projected on to a two dimensional plane and volumes were estimated from the projected area. As expected, the absolute volumes deduced from the two methods were different but the normalized results were identical (data not shown). This procedure allowed us to take multiple measurements of the same nucleus using two

dimensional imaging without the photobleaching associated with repeated three dimensional imaging. In contrast to nuclei inside intact cells, isolated nuclei sit directly on the glass coverslip so they are constrained on one side. In this case, it was considered appropriate to image the volume directly using stacked confocal imaging and design the experiment to rely on only one pair of stacked images.

The physiological implications of changes in nuclear morphology are not fully understood, but they are observed in association with a number of important biological phenomena. Genome organization influences a host of fundamental biological processes^{50, 51} and is presumably altered by changes in nucleus morphology. There is evidence that changes in nuclear shape and volume may alter nucleo-cytoplasmic transport. For example, nuclear permeability is increased in flattened cells as compared to rounded cells, and increased tension in the nuclear envelope is one mechanism that has been proposed for this phenomenon²⁸. A rough nuclear perimeter is observed in cases where lamin is defective due to a genetic disease such as Hutchinson-Gilford progeria syndrome (HGPS)³¹, supporting the idea that lamin-dependent membrane mechanics in the nuclear cortex maintain the conventional smooth shape of the eukaryotic nucleus. Despite the devastating consequences of the disease, the nuclei of HGPS cells are not more fragile than those of healthy cells but they do exhibit reduced lamin mobility and altered lamin organization²¹. This finding suggests that the nuclear lamina is much more than a passive structural element and that reorganization of the lamina under load is a sensitive and important mechanotransduction process. The smoothness of the nuclear perimeter also deteriorates in normal aging⁶⁵, increasing the relevance of these changes to degenerative diseases such as osteoarthritis. While both normal aging and HGPS reduce the capacity of the nucleus to repair DNA, it is not known what connection, if any, the physical changes associated with these conditions have to DNA repair. However, it is worth noting that exposure to a hyper-osmotic environment, which is shown here to produce a rough nuclear perimeter, also inhibits DNA repair²⁴. The role of cause and effect may be interchangeable between biological and physical phenomena in some cases. For example, cell shrinkage and chromatin condensation are traditionally viewed as effects of apoptosis but hyper-osmotic shrinkage sensitizes hepatocytes towards CD95 ligand-induced apoptosis⁶⁰.

In summary, the findings of this study show that nucleus morphology is osmotically sensitive and the relationship between morphology and osmolarity is significantly non-linear, in contrast to the behavior of the cell. The nuclear response does not depend on integrity of the actin cytoskeleton. Isolated nuclei contract in response to physiologic changes in macromolecule concentration but not in response to similar changes in the concentration of common intracellular ions, suggesting that osmotic pressure arises from size exclusion of large solute molecules rather than membrane confinement of ions. These results on passive cells also offer a valuable baseline for studies that seek to interpret physical changes that occur during active cell processes.

Materials and Methods

Mathematical Model

The equilibrium volume response of the nuclei was modeled using an extension of an existing model of the swelling of porous gels² to estimate the stiffness of the nuclear lamina. The original model describes a spherical gel without a surface membrane. The permeation of the solute molecule into the pore fluid is limited by steric exclusion. This is quantified using the partition coefficient, κ , which establishes the following relationship (eqn. 14 in Albro et al.²) between the concentration within the gel, c_{eq} , and the concentration in the surrounding bath, c^* .

$$c_{eq} = \kappa c^* \quad (1)$$

The partition coefficient is determined of the pore size distribution in the gel and the size of solute molecule. The partition coefficient only departs from unity in the case of the large solute molecules for which steric exclusion effects are non-negligible. In the case of the cell nucleus, the surrounding bath is the cytoplasm and it contains many solute species, each of which has a different partition coefficient between the cytoplasm and the nucleoplasm so it is necessary to define an equivalent partition coefficient. Note that:

$$c_{cyt} = c^* \quad (2)$$

for a cell that behaves as a perfect osmometer where c_{cyt} is cytoplasmic concentration and c^* is the extracellular concentration.

For i species of cytoplasmic solute,

$$\text{For } i \text{ species of cytoplasmic solute, } c_{cyt} = \sum_i c_{cyt}^i = \sum_i f^i c^* \quad (3)$$

where f^i is a constant characteristic of each species and independent of osmotic expansion or contraction of the cell because only water crosses the cell membrane.

Each cytoplasmic species will partition into the nucleoplasm according to its partition coefficient

$$c_{nuc}^i = \kappa^i c_{cyt}^i \quad (4)$$

$$c_{nuc} = \sum_i \kappa^i c_{cyt}^i = \sum_i \kappa^i f^i c^* = \kappa_e c^* \quad (5)$$

where the equivalent partition coefficient, $\kappa_e = \sum_i \kappa^i f^i$ and is a constant regardless of osmotic expansion or contraction.

The equivalent partition coefficient of the chondrocyte nucleus can be determined from the hyperosmotic contraction using equation 15 in Albro et al.

$$H_a \left[\frac{\partial u}{\partial r} \Big|_{r=a} + \frac{2\nu}{1-\nu} \frac{u(a,t)}{a} \right] = -R\theta(1 - \kappa_e)c^*(t) \quad (6)$$

where H_a is aggregate modulus, u is solid matrix displacement, r is the radial coordinate, a is the radius in a zero osmolarity bath, ν is the Poisson's ratio, t is time, R is the universal gas constant and θ is the absolute temperature. Substituting in the displacement solution $u(r,t) \rightarrow$

$\infty) = \zeta r$ and the relation between bulk modulus, K , and aggregate modulus $K = H_A(1 + \nu)/3(1 - \nu)$ yields the following relation

$$3K\xi = -R\theta(1 - \kappa_e)c^* \quad (7)$$

Writing volume in terms of ζ and neglecting higher order terms because this is a small strain analysis leads to the following expression 20 in Albro et al. relating the normalized volume to the extracellular osmolarity.

$$\frac{V_{eq}}{V_r} = \frac{1 - R\theta(1 - \kappa_e)c_{eq}^*/K}{1 - R\theta(1 - \kappa_e)c_r^*/K} \quad (8)$$

where subscript r refers to the reference condition, 380 mOsm in this experiment. This allows determination of κ_e from the hyperosmotic response if K is specified. Most of the mechanical models of the cell nucleus in the literature model it as an incompressible material and therefore that data cannot be used to determine K . However, one investigator reported large volumetric strains under pressure in micropipette experiments 63. While the data was not presented in a form that allows exact determination of K , the range of pressures applied and strains measured suggests that a value of 10 kPa is accurate to within one order of magnitude. More accurate measurements of this property in the future would increase the utility of this model. In this analysis, a single data point corresponding to an extracellular osmolarity of 482 mOsm was used to evaluate κ_e on the grounds that the model was originally developed using small strain assumptions violated at higher osmolarities. The strain at 482 mOsm is the only hyperosmotic strain that is within the range for which the model was originally validated. The value of κ_e determined by this approach was 0.9956.

The hypoosmotic response of this model with respect to inverse normalized osmolarity is nonlinear. However, the non-linearity is not strong enough to fit the trend in the observed data. This, combined with the observed changes in shape during hypoosmotic expansion, supports the introduction of a membrane stress term. Equation 7 is a relation between volumetric strain and pressure. The membrane tension is represented as a pressure term in equation 7 using the Law of Laplace ⁴ to create the following equation:

$$3K\xi = -R\theta(1 - \kappa_{eq})c^* - \frac{2T}{q} \quad (9)$$

$$\text{where } T = \begin{cases} 0, A < A_{crit} \\ K_A \left(\frac{A}{A_{crit}} - 1 \right), A > A_{crit} \end{cases} \quad (10)$$

where T is membrane tension, q is the radius, K_A is the dilatational modulus of the membrane, A is the membrane area and A_{crit} is the critical area at which the membrane is drawn taut. Fitting the governing equations (9) and (10) to the data presented in Fig. 1A allows determination of the mechanical properties of the nuclear lamina, specifically the K_A and c_{crit} , the osmolarity at which the nuclear membrane begins to resist further osmotic expansion (the critical value of the osmolarity is determined from the corresponding value of ζ using equation 7). To solve these equations, the area must be written in terms of ζ as follows:

$$A = 4\pi q^2 = 4\pi a^2(1 + \zeta)^2 \approx 4\pi a^2(1 + 2\zeta)$$

$$A_{crit} \approx 4\pi a^2(1 + 2\zeta_{crit}) \quad (11)$$

and substituted into equation 9. For $A < A_{crit}$, ζ is related to c^* by equation 7. For $A > A_{crit}$, the resulting form of equation 9 was solved in Mathematica (Wolfram Research Inc., Champaign, IL) to yield an analytical expression for ζ . Albro et al.'s equation 17 can be used to give the normalized volume in terms of ζ as follows

$$\frac{V_{eq}}{V_r} = \frac{1 + 3\zeta}{1 - R\theta(1 - \kappa)c_r^*/K} \quad (12)$$

This model was coded in Matlab and fit to the hypoosmotic volume data numerically.

Cell isolation and culture

Chondrocytes were isolated from articular cartilage of the lateral femoral condyle of 2–3 year old skeletally mature female pigs obtained from a local abbatoir. Cells were isolated by sequential digestion in pronase and collagenase solution and seeded on glass coverslips in Dulbecco's modified Eagle medium containing 10% fetal bovine serum, 1.5% HEPES and 1% non-essential amino acids (all cell culture reagents were supplied by Gibco, Grand Island, NY). The medium was adjusted to pH 7.4, and its osmolarity was increased to 380 mOsm with sucrose and verified with a freezing point osmometer (Precision Systems Inc., Natick, MA). 380 mOsm is an approximate value for the extracellular osmolarity experienced by chondrocytes *in situ* 71. For confocal microscopy experiments, 0.2 ml of media containing approximately 2×10^5 cells was spread on the center of a 42mm diameter coverslip that was placed in a 60 mm diameter culture dish. Cells were incubated for 1 hour to allow attachment to the coverslip, and 5 ml of media was added to each dish before incubation was continued. For angle resolved low coherence interferometry (a/LCI) experiments, chondrocytes were seeded at a density of $7 \times 10^5/\text{cm}^2$ in chambered coverglasses. Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂ for 18 hours. As a result, the chondrocytes were attached to the glass but maintained their rounded morphology at the time of the experiments²⁶.

Microscopic Imaging

Each coverslip was incubated in 380 mOsm saline with 10 μM acridine orange (Molecular Probes, Eugene, OR), a fluorescent label of nucleic acids, for 20 minutes to label the nuclei. The cells and their nuclei were visualized using confocal laser scanning microscopy (LSM 510, Carl Zeiss, Thornwood NY). Images were obtained through an inverted fluorescent microscope (Axiovert 100M, Carl Zeiss) with a C-Apochromat, 63x, water immersion, 1.2-NA objective lens. Acridine orange fluorescence was excited by an argon-ion laser (488 nm) at 2% power and the stimulated emission was collected through a 505–550 nm filter on an 8-bit intensity scale. For DIC images, the sample was also illuminated with a helium-neon laser at 80% power. Images of 1024×1024 pixels were recorded with a scale length of 0.12 microns per pixel. The confocal pinhole was fully open, creating an optical slice thickness in excess of 7.5 μm . For 3 dimensional imaging of isolated nuclei, the step size between images was 0.5 μm and samples were incubated with Syto 13 (Molecular Probes, Eugene, OR) for 30 minutes to fluorescently label nuclei acids. Other settings were identical to those used for two dimensional imaging.

Angle resolved low coherence interferometry (a/LCI) experiments

A/LCI is a non-perturbative optical technique that measures the average nuclear size of cells in a biological sample, such as tissues or, in the case of the present study, on isolated cells. A/LCI combines the capabilities of low coherence interferometry with light scattering techniques to determine nuclear morphology with subwavelength accuracy^{13, 14}. Low coherence interferometry uses a wide bandwidth source for the purposes of achieving high depth resolution and rejecting multiply scattered light in a signal, as in optical coherence tomography³⁸. This permits the ability to make sensitive measurements of light scattered from a single cell monolayer. Inverse light scattering analysis (ILSA) is predicated on the fact that elastically scattered light from an object yields a unique signature that is a function of the shape, size, and electromagnetic properties of the object. In the case of a/LCI, the angular light scattering distribution of a population of cells is processed to isolate the nuclear scattering contribution and compared to an appropriate model⁵⁹. Upon statistical comparison to the model, the characteristics of the scattering objects, in this case nuclear size, can be discerned. The nuclear size of a monolayer of chondrocytes cultured in a chambered coverglass was measured after equilibration to saline at a range of different osmolarities.

Equilibrium effects of osmolarity on cell and nuclear morphology

The coverslip was loaded into a perfusion chamber (PeCon GmbH, Kornthalde, Germany) and washed three times with saline at 380 mOsm. The chamber was mounted on the microscope stage and 9 consecutive sequences of images were recorded, each consisting of 25 DIC images at 5 second intervals followed by a single fluorescent image. At the beginning of each of these sequences, the saline in the chamber was withdrawn and replaced with saline at a different osmolarity, starting with the highest value and proceeding to the lowest. The osmolarities used are listed in Table 1.

Transient effects of osmolarity on cell and nuclear morphology

Samples were washed and mounted in the perfusion chamber on the microscope stage as described above. A single scan, including DIC and fluorescent images, was recorded. The saline in the chamber was then exchanged and 9 further scans were taken over the next 140 seconds at irregular time intervals so that imaging would be most frequent during the most dynamic portion of the response. The saline was exchanged with saline at 280 mOsm for hypo-osmotic transients and 480 mOsm for hyper-osmotic transients. In the transient experiments, size is quantified using cross-sectional area.

Calculation of time constants of cross-sectional area change

The following equation:

$$A(t) = 1 + K(1 - e^{(-t/\tau)}) \quad (13)$$

was fit to aggregated data points from multiple experiments using non-linear regression to calculate the time constant of the response. In this expression, $A(t)$ is the normalized cross sectional area, K is the amplitude of the response, t is time and τ is the time constant. Non-linear regression was performed using Matlab to determine values and 95% confidence intervals for the amplitude and time constant. Values are reported to be significantly different if the confidence intervals do not overlap.

Osmotic loading of cytochalasin D treated chondrocytes

Chondrocytes were isolated and seeded on glass coverslips as described above. Cytochalasin D-treated chondrocytes were incubated with 2 μ M cytochalasin D in DMEM with 10% FBS

for 3 hours before testing while untreated chondrocytes were not. Both sets of chondrocytes were stained for 30 minutes with 0.5 μM Syto 13 (Molecular Probes, Eugene, OR) before testing to fluorescently stain the nuclei. For testing, the coverslip was mounted in the perfusion chamber, washed three times in 380 mOsm media and mounted on the microscope stage. A single two-dimensional fluorescent image was taken before any fluid exchange to serve as a reference. Then, the 380mOsm media in the chamber was exchanged with fresh 380 mOsm media and a second image was taken to serve as an iso-osmotic control. Finally, the 380 mOsm media was exchanged with 580 mOsm media and a third image was taken. Two minutes was allowed for equilibration after each exchange before an image was taken.

Response of isolated nuclei to elevated concentrations of macromolecules and ions

Nuclei were chemically isolated from freshly isolated chondrocytes as described previously³⁵. Briefly, 10 million freshly isolated porcine chondrocytes were suspended in 0.1% IGEPAL CA-630 for 8 minutes at 4° C. The suspension was diluted 10:1 with 0.75% BSA in HBSS (both from Gibco, Grand Island, NY) and centrifuged at 500g for 20 minutes at 4° C. The pellet was resuspended in 0.75% BSA in HBSS and 100 μl droplets were spread on 42 mm coverslips and allowed to seed for 1 hour. Then, the coverslips were covered in iso-osmotic testing buffer and incubated overnight before testing. The iso-osmotic testing buffer was 15 mM HEPES, 10 mM NaCl, 140 mM KCl, 500 μM MgCl_2 and 100 nM CaCl_2 . These ion concentrations are typical of those in a mammalian cell at equilibrium⁹. For testing, the coverslips were mounted in the perfusion chamber, washed three times in iso-osmotic testing buffer and mounted on the microscope stage. To simulate the crowded environment of the cytoplasm, the buffer was exchanged for a 25% solution of 10 kDa dextran (Sigma-Aldrich, St. Louis, MO) in iso-osmotic testing buffer, allowed to equilibrate for 2 minutes and imaged in three dimensions using the confocal microscope. For the control condition, the fluid was then withdrawn from the chamber and replaced with an identical solution. For the high macromolecule condition, the fluid in the chamber was exchanged with a 34% dextran solution in iso-osmotic testing buffer. This condition models the increase in macromolecular crowding that would arise from an abrupt 26% decrease in the cell volume, as is typical of chondrocytes subjected to 380 mOsm – 580mOsm hyper-osmotic step change (see Results). In the high ion condition, the fluid was exchanged with a 25% dextran solution in a high ion buffer consisting of 15 mM HEPES, 13.5 mM NaCl, 189 mM KCl, 676 μM MgCl_2 and 135 nM CaCl_2 . These ion concentrations model the increase in ion concentration that would arise from an abrupt 26% decrease in the cell volume.

Image Analysis

Chondrocyte volume was determined using an edge detection algorithm applied to the DIC images as described previously³. Briefly, the focal plane of the microscope was manipulated during the experiment to create a diffraction pattern in the form of white ring marking the edge of the cell (Fig. 4). A custom-written application in PV-WAVE software (Visual Numerics, Inc., Houston, TX) was used to define an annular region around the cell containing the ring and apply Sobel edge detection and optimization algorithms to calculate a closed curve representing the edge of the cell. This allowed direct measurement of the cross-sectional area from which volume was inferred by assuming the cell to be spherical. The cross-sectional area, perimeter length and contour ratio of the nucleus were determined from the fluorescent images using Otsu segmentation and the Image Analysis Toolbox in Matlab (The MathWorks, Inc., Natick, MA). The contour ratio of a shape is defined as $4\pi \times \text{area/perimeter}^{2,31,45}$. The nucleus volume was also determined from the cross sectional area by approximating the shape to be a sphere. For isolated nuclei, raw stacked images were deconvolved using commercial deconvolution software Huygens (Scientific Volume Imaging, Amsterdam, the Netherlands). The deconvolved image was exported to Matlab and thresholded using the Iterative Self Organizing Data algorithm to determine the volume.

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Figure 1.

(A) The Ponder's relation (inverse osmolarity vs. normalized volume) was found to be linear for the cell but nonlinear for the nucleus. The non-linearity of the response of the nucleus was independent of the techniques used to measure nuclear size (i.e., fluorescent microscopy or angle resolved low coherence interferometry (a/LCI)). For nucleus by a/LCI, $n = 11$. For cell and nucleus by microscopy measurements, n is as per table 1. Error bars represent one standard error. V/V_{iso} = volume/iso-osmotic volume, C_{iso}/C = iso-osmotic osmolarity/osmolarity. (B) Values of the slope of the Ponder's plot calculated for the cell and the nucleus (as measured by fluorescence microscopy) in the hyper- and hypo-osmotic range (error bars denote the 95% confidence interval). Bars with different letters are significantly different. The observed

differences are protected by a two way ANOVA, which returns a significant main effect of structure (cell versus nucleus), no significant main effect of osmotic range (hyper-osmotic or hypo-osmotic) and a significant interaction between structure and range. Slopes were considered different to statistically significant degree if the 95% confidence intervals of the slopes do not overlap. Bars labeled with different letters are statistically significantly different to one another.

Figure 2. Observed nuclear volume data from 480 mOsm to 200mOsm along with the model for the same osmotic range with best-fit membrane properties and bulk modulus of 10 kPa.

Figure 3.

In the nucleus, the relationship between osmolarity and contour ratio followed a similar trend to the relationship between osmolarity and volume at equilibrium. Error bars represent one standard error.

Figure 4.

Typical images of a single cell (top) and its nucleus (bottom) during equilibrium experiments at a range of osmolarities. The number above the each image pair indicates the osmolarity in mOsm. The number below each pair of images indicates the inverse normalized osmolarity.

Figure 5.

Cell and nucleus cross sectional area transients showed rapid first order dynamics with no evidence of recovery. (A) The means and standard errors of the cell transient response along with the best fit of Eq. 13 and the 95% confidence interval ($n = 46$ and 45 for hypo-osmotic and hyper-osmotic tests respectively). (B) The means and standard errors of the nucleus transient response along with the best fit of Eq. 13 and the 95% confidence interval ($n = 39$ for both the hyper-osmotic and the hypo-osmotic tests). Note that means are presented for illustrative purposes only and the actual fit was to the whole population of data points rather than to their means.

Figure 6.

The shape changed more slowly than the size. To accommodate this, the perimeter was extended at early time points before recovering as the shape evolved into a smoother profile. Error bars represent one standard error ($n = 39$).

Figure 7.

The morphological effect of hyper-osmotic loading is not influenced by cytochalasin D treatment. (A) The nuclear volume is reduced by approximately 26% in both the untreated and cytochalasin D treated case. (B) The nuclear contour ratio is increased by approximately 5% in both the untreated and the cytochalasin D treated case, $n = 34$ nuclei per group, error bars represent one s.d., * indicates statistically significant difference.

Figure 8.

Isolated nuclei contracted in response to increased macromolecule concentration but not in response to increased ion concentration, $n = 100-126$, error bars represent one s. d., * indicates statistically significant difference. “Hyper ions” denotes elevated ion concentration. “Hyper macro” denotes elevated macro molecule concentration (see Materials and Methods for details).

Figure 9.

(A) Merged DIC and rhodamine fluorescence image of isolated nuclei incubated with rhodamine-labeled 10 kDa dextrans at room temperature. (B) Plot of intensities of DIC image and rhodamine fluorescence along in the green line in (A). There is almost no difference in the intensity of rhodamine staining inside and outside the isolated nuclei.

Table 1

Number of samples used in equilibrium experiments.

Osmolarity (C)	Inverse Normalized Osmolarity (C_{iso}/C)	Number of cells	Number of nuclei
906	0.419	69	17
717	0.530	69	26
586	0.649	69	36
482	0.788	69	39
378	1.005	69	52
280	1.357	64	44
237	1.603	58	38
215	1.767	39	26
200	1.900	27	24

C_{iso} refers to the iso-osmotic osmolarity, 380 mOsm.

Table 2

Time constants in seconds along with 95% confidence intervals for best fits to transient responses.

	Hypo-osmotic	Hyper-osmotic
Cell	9.838 ± 1.013 (n = 46)	5.670 ± 0.939 (n = 45)
Nucleus	7.800 ± 3.924 (n = 39)	7.245 ± 1.788 (n = 39)