Perspectives on: The response to osmotic challenges

Cell volume control in three dimensions: Water movement without solute movement

Frederick Sachs[1] and Mettupalayam V. Sivaselvan[2]

[1]Department of Physiology and Biophysics and [2]Department of Civil, Structural and Environmental Engineering, University at Buffalo, The State University of New York, Buffalo, NY 14260

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Cell volume primarily represents the amount of water in a cell. Osmotically active metabolites and mechanical forces can cause changes in cell volume, but cell volume is under feedback control around a characteristic set point. Traditionally, the analysis of cell volume regulation treats the cell as a semipermeable bag with osmotic pressure converted to hydrostatic pressure at the membrane. However, newer data show that in cells with a cross-linked cytoskeleton, osmotic stress is distributed throughout the cell volume and not confined to the cortex. Cytoskeletal cross-linking creates a sponge-like interior bonded to the membrane at the periphery. The cytoskeleton can be strong enough to allow a cell to withstand hours-long exposure to distilled water without lysing. The elastic energy stored in a swollen, cross-linked cytoskeleton is much larger than that in the cell cortex (see Appendix) and hence is a critical variable to include when modeling cell volume regulation.

Traditional analyses of cell volume regulation have used a Donnan equilibrium model that treats the cell as a semipermeable bag containing mobile and immobile ions (Hill, 1956; MacGillivray, 1968; Ricka and Tanaka, 1984). However, electrostatics are not the only forces at play; missing is the elasticity of the cytoskeletal matrix (Rice, 1998; Wang, 2000b; Charras et al., 2009; Moeendarbary et al., 2013). The physics of this "poroelastic" system is well understood (Biot, 1941; Hill, 2012) but has usually not been incorporated into discussions and models of osmotic balance and cellular volume changes.

Poroelasticity describes the interaction between fluid flow and solid deformation within a porous medium. When an external load is applied sponge, the fluid filled pores of the sponge experience a change in pressure, and this leads to fluid flow causing deformation of the elastic skeleton of the sponge. Poroelasticity is a common model for inhomogeneous materials containing fluids. This includes cells (Mitchison et al., 2008), bone (Cowin, 1999), collagen (Chandran and Barocas, 2004), and soil (Deresiewicz and Skalak, 1963; Wang, 2000b). Modeling poroelasticity combines two laws: Darcy's law describes the fluid motion and pressure in a porous medium (Whitaker, 1986) and states that the fluid velocity is proportional to the pressure gradient, the fluid viscosity, and the material's ability to disrupt the flow. The second law describes the mechanics of the matrix under the combined action of forces in the sponge network and the hydrostatic pressure in the pores. Biot (1941) merged these two laws. In the Appendix, we present an analysis of a swollen cell, demonstrating that the elastic energy stored in the cytoskeleton is much larger than that in the cell cortex.

For the time being, let's forget the cell membrane and consider a membrane-free preparation: a sponge in water. The sponge starts out small and stiff, and then soaks up water, expands, and gets softer (Rey and Vandamme, 2013). It continues to expand and then reaches equilibrium without lysis. Clearly the swelling and softening are not dependent on the chemistry of the sponge, as the same thing happens with noodles or wood (Rand, 2004), and there is a large transfer of water without mobile solutes, and sponges don't lyse. To quote Charras et al. (2009): "an internal gradient in hydration is inconsistent with a continuum model for cytoplasm, but consistent with the sponge model." Cells, like sponges, are made of wettable cross-linked polymers, and the thermodynamics suggests that there must be similarities in water transport (Hill, 2012). Let's return to our sponge in some more detail.

Why does the sponge swell? At the beginning, water sticks to the hydrophilic polymers that form the sponge—a restatement of the fact that the sponge is wettable. However, after a few water molecules stick, the additional water starts looking like bulk water (Parsegian et al., 1987). Yet water keeps entering the sponge (you might consider a half-filled sponge compared with a filled sponge). Water diffuses (or initially flows) into a sponge

Correspondence to Frederick Sachs: sachs@buffalo.edu

Abbreviations used in this paper: AFM, atomic force microscopy; BAEC, bovine aortic endothelial cell; MSC, mechanosensitive ion channel.

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because there is room for it, and the second law of thermodynamics applies. But why does the sponge stop swelling and reach equilibrium?

Equilibrium means that the free energy to transport a water molecule from the sink to the sponge is the same as the free energy to transport a water molecule from the sponge to the sink. To transfer a water molecule into a sponge you have to make room for it. But water in the sponge is surrounded by elastic threads, and adding water requires that you stretch those threads. Stretching the polymers requires mechanical work, and that elastic energy squeezes the enclosed water, increasing its hydrostatic pressure. The equilibrium is reached when the entropic energy $T\Delta S$ for water diffusion into the sponge is equal $P\Delta V$, where P is the hydrostatic pressure of water in the free volume, ΔV, in the sponge. The hydrostatic pressure of water in the sponge is higher than the water pressure in the sink. An interesting prediction for cells that has been tested on inanimate systems is that stretching a sponge causes a water influx (Hill, 2012).

Let's look at a picture of a sponge (Fig. 1). The figure shows open (water-filled) regions surrounded by filaments, and like all materials, the filaments are elastic. If we want to transfer water into the open regions of the sponge, we have to expand them. To do that, the boundary filaments must stretch, and those springs store an energy of $\Delta G = kx^2/2$, where k is the stiffness of the filament and x is the displacement from rest. To store more water, we need to stretch the filaments, and that takes energy and that comes from $T\Delta S$, the driving force of diffusion. Squeezing the boundaries of a volume of water in the sponge increases its hydrostatic pressure, and it is this pressure that resists the influx of more water. This is true no matter what the sponge is made of (Rand, 2004; Trombetta et al., 2005), and the cytoskeleton does look like a sponge (Fig. 2; Xu et al., 2012). If the sponge fibers had an adjustable stiffness, we could make the sponge pump water without requiring the transmembrane movement of any mobile solutes, and we know that the cytoskeleton can contract and relax (Taber et al., 2011). A quantitative comparison of the bulk stress and the cortical stress is addressed in the Appendix.

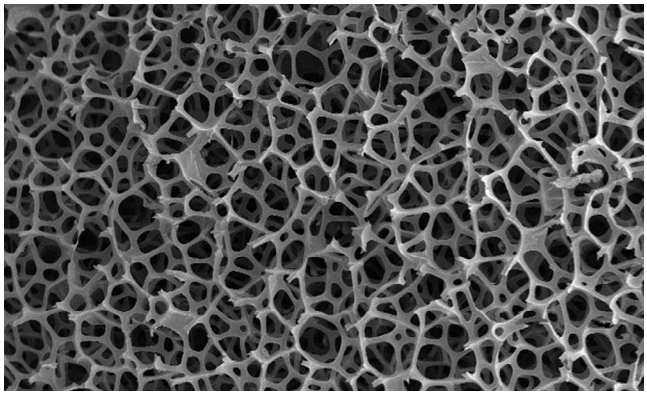

Figure 1. SEM image of a sponge structure. The spatial units are arbitrary. In some sponges, filaments may be under low tension so that their elasticity is entropic rather than enthalpic. (Courtesy of Janice Carr, Centers for Disease Control and Prevention)

Did nature ignore physics when it had to deal with cell volume regulation? Using optical probes to measure the stress in the structural proteins of cells, we found that with an osmotic challenge, mechanical stress is distributed in three dimensions throughout the cytoskeleton and not concentrated at the cortex like a balloon (Meng, 2008; Spagnoli et al., 2008; Meng and Sachs, 2012; Guo et al., 2014). If the cell membrane separates from the cytoskeleton, it behaves with two-dimensional mechanics like red cells (Savitz et al., 1964), lipid vesicles (Kwok and Evans, 1981; Evans and Needham, 1986, 1987), and balloons.

I suspect we were all taught that the cell membrane is responsible for the control of cell volume, but that was prompted by red cell data and ignored the behavior of cells with a space-filling cytoskeleton (Hoffman and Crocker, 2009). We were also told that cells cannot be put in distilled water because they would lyse. We had not bothered to test that dogma until pressed to do so by conflicts generated by our data. We did the test and found that it is generally incorrect. Many cells can live in distilled water for hours (Wan et al., 1995; Meng and Sachs, 2012; Guo et al., 2014). How can they do that?

For cells placed in distilled water, the osmotic pressure gradient can be predicted by the Morse equation $\Pi = iMRT$, where i is the Van 't Hoff factor representing the activity coefficient, M is the mobile solute concentration, R is the gas constant = 8.3 J/mol · K, and T is the absolute temperature. Given the intracellular concentration of diffusible solutes, we predict that a cell in distilled water would initially feel Π of ~6 atm of hydrostatic pressure across the membrane, about twice the pressure in a car tire. But we have shown that cells need not lyse under these conditions. Why not?

The cytoskeleton inserts components into the bilayer, creating a lattice of ~30 nm (Bovellan et al., 2014). The bilayer is thus divided into small regions with a small radius of curvature (Fig. 3). Laplace's law states that the tension in a spherical cap with radius of curvature r is given by $\gamma = P\,r/2$, where P is the transmembrane pressure. The smaller the radius of curvature, the smaller the tension (Suchyna et al., 2009). The cytoskeleton creates a small radius of curvature (Huang et al., 2013) so it feels little tension at a given hydrostatic pressure.

Bovine aortic endothelial cells (BAECs) survive for hours in distilled water. Why should they have evolved that capability? BAECs evolved to line the blood vessels where the shear stress of blood flow on the apical side tries to pull the cell downstream, whereas adhesion plaques on the basal side keep the cell from blowing away. The cytoskeleton must firmly attach the apical to the basal side of the cell to resist that shear stress (Heo

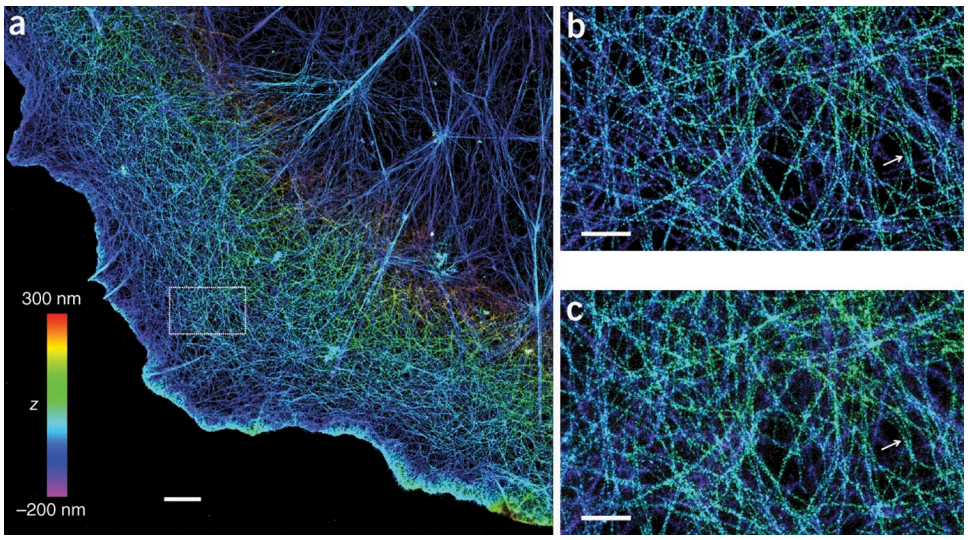

Figure 2. Image of the actin cytoskeleton in a COS-7 cell showing the free volumes contained in the "sponge" of the cytoskeleton. Panels b and c are zoomed versions of the box in a. The colors code the distance of the actin from the substrate. These images are made using only labeled actin, and there are many other proteins that make up the cytoskeleton, so the protein density and the free volumes are smaller. Bars, 2 µm (Xu et al., 2012). Reprinted by permission from Macmillan Publishers Ltd: *Nature Methods*. 9:185–188. 2012. Intermediate filaments bear a lot of stress (Fudge et al., 2008).

et al., 2012). This same structure provides the support that allows those cells to survive in distilled water for hours (Meng et al., 2008). Hochmuth's laboratory measured cortical stiffness using aspiration and found that endothelial cells and chondrocytes behaved as elastic solids, not floppy membranes like neutrophils and red cells (Discher et al., 1994; Hochmuth, 2000). Morris's laboratory studied the effect of extreme osmotic stress on molluscan neurons and showed that they too can survive in distilled water (Wan et al., 1995). Nucleated cells that have a cross-linked cytoskeleton are rarely spherical (Stewart et al., 2011), and thus they should not behave mechanically like spheres. There are significant forces in the cytoskeleton pulling normal to the cell surface (Fig. 2), and they define the cell shape. Those forces come from the cortical cytoskeleton pulling against the deeper cytoskeleton and the substrates. If the cortex should separate from the deeper cytoskeleton, as it does in blebs (Charras et al., 2005; Moeendarbary et al., 2013), the cortex becomes stiff because it is under a hydrostatic pressure gradient (Beyder and Sachs, 2009, 2011). We found that in HEK cells, about half of the cortical stress is in the bilayer and half in the attached proteins (Akinlaja and Sachs, 1998).

When we perform patch-clamp experiments on mechanosensitive ion channels (MSCs), we typically apply a pipette suction of 20–50 mmHg to stretch the patch and activate the channels (Guharay and Sachs, 1984). We tried many times to activate MSCs with osmotic pressure of much greater magnitude and usually failed, as have others (Morris and Horn, 1991). Why do channels respond to pressure in patches but not in cells?

I asked my postdoc, Dr. Chiara Spagnoli, who was fluent in atomic force microscopy (AFM) to see how stiff

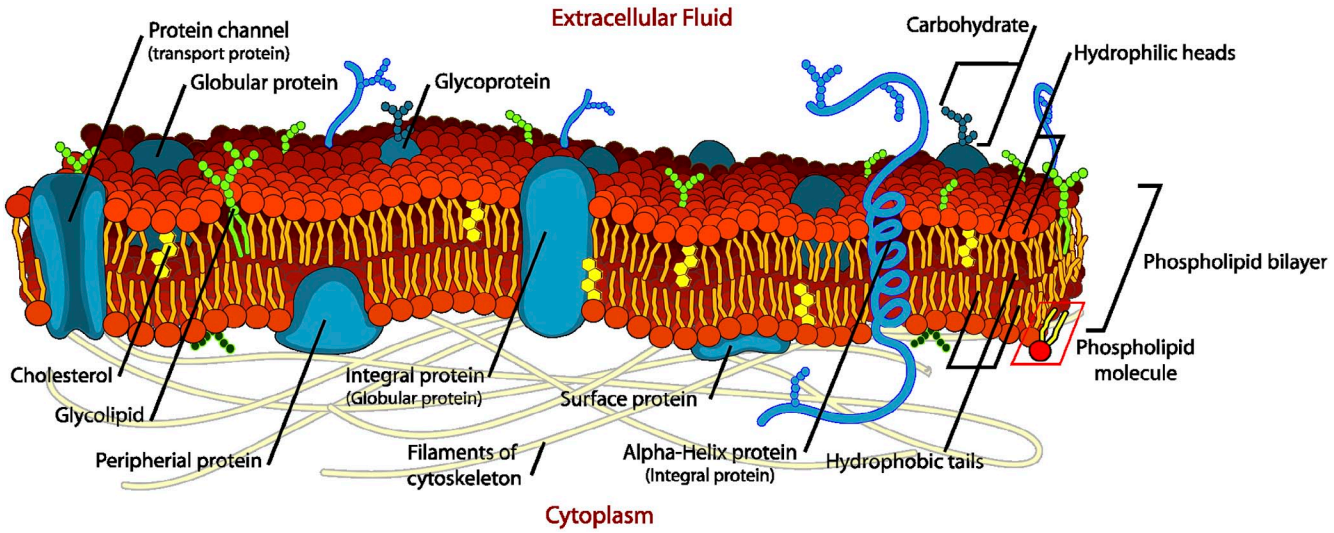

Figure 3. Cartoon emphasizing the curvature of the bilayer in cells that makes it much more resistant to pressure-induced lysis than a planar membrane. (Courtesy of *Lady of Hats [Mariana Ruiz], Wikipedia Commons*)

cells become during hypoosmotic swelling, postulating the dogma that they should get stiffer as they inflate. However, after months of testing and many controls, she found that the cells stayed the same or got softer with swelling (Spagnoli et al., 2008). After much agonizing, we realized that the behavior was much like how sponges behave. The reason we don't often see MSCs activated with osmotic pressure is because the membrane isn't stretched very much (Sachs, 2015); the primary stresses are internal to the cell and not concentrated in the cortex. Although the cytoskeleton has been suggested previously to be involved in cell volume regulation based upon the effect of cytoskeletal reagents, the literature has lacked a unified explanation as to how and why that should occur (Mills and Skiest, 1985; Strange, 1993; Williams et al., 1997; Lang and Hoffmann, 2013). Most papers on cell volume regulation emphasize the role of the membrane and ignore the role of the cytoskeleton (Kregenow, 1981; Wehner et al., 2003; Hoffmann et al., 2009). Is the membrane involved in volume regulation?

Long ago, MSCs were suggested as possible sensors for cell volume regulation (Chamberlin and Strange, 1989; Sachs and Morris, 1998). To test this hypothesis, we inhibited MSCs in intact cells with GsMtx4, the only known specific inhibitor (Bowman et al., 2007; Bae et al., 2011), and measured cell volume regulation (Hua et al., 2010). The result was a bit disappointing for those of us who love MSCs; most cell types do not use MSCs for volume regulation, but some cells, such as NRK cells (neonatal rat kidney), do use them (Hua et al., 2010). Thus, nature has developed multiple ways to deal with the universal problem of cell volume regulation. (Researchers should beware that cytoskeletal structures, and hence their stress, can be altered simply by expression of proteins that may include nonconducting ion channels; Lauritzen et al., 2005.)

The sharing of stress between the membrane and the cytoskeleton marked a major split in evolution. I suggest it stemmed from the requirement of animal cells to handle osmotic pressure without a cell wall. The walled cells, like bacteria, embed their metabolism in a semipermeable membrane that is enclosed in a rigid container that can withstand large hydrostatic pressures (Martinac et al., 2014). However, for animal cells to evolve and become mobile, the cell wall had to be eliminated, but that would have led to membrane lysis (Kung, 2005; Kung et al., 2010). To avoid lysis, the animal cells evolved an internal skeleton to resist the hydrostatic pressure (Spagnoli et al., 2008), and this skeleton was also made dynamic to allow the cells to be motile (Lieber et al., 2013; Martinac, 2014).

The AFM experiments described above predicted that if we were to measure the stress in the cross-linked cytoskeleton of cells subjected to hypotonic stress, we would find it distributed throughout the cell and not confined to the cortex. That is what we found (Meng et al., 2008; Meng and Sachs, 2012; Guo et al., 2014). We made genetically coded optical probes to report the tension in chimeric structural proteins such as actin, actinin, spectrin, and filamin (Meng and Sachs, 2012; Guo et al., 2014), and expressed the chimeras in a variety of cell types including HEK, MDCK, 3T3, and BAEC. Hypotonic challenges led to swelling as expected, but the stresses were distributed throughout the cell and not concentrated in the cortex. Thus, osmotically induced stresses are primarily a bulk property of cells and not confined to the cortex.

Because the mechanical cortex is so thin, it is difficult to measure stresses in the cortex without contamination with the deeper cytoskeleton. Zou et al. (2013) performed AFM/cell volume experiments and found that ionic fluxes through channels could change cell stiffness, but those changes were different from applying osmotic pressure alone. They attributed their stiffness changes to stiffening of the proteins in the cortex, but they did not measure the contribution of the deeper cytoskeleton. The cortex is <0.2-µm thick (Clark et al., 2013), and because the AFM indentations were on the order of 1 µm, the deeper cytoskeleton was also deformed (Johnson, 1987). The same problem applies to the work of Stewart et al. (2011) and Fischer-Friedrich et al. (2014). A key feature of the cytoskeleton that affects volume regulation is that it is cross-linked. In muscle, the cytoskeleton is not heavily cross-linked because it needs to move freely, and hence the poroelastic contribution to cell volume is minor and will tend to make the cell behave closer to a "perfect" osmometer (Hodgkin and Horowicz, 1959). This would suggest that the osmotic response of living cells and cells in rigor mortis should be different. But what about the membrane?

Most of the world's literature on cell volume regulation suggests that the membrane is the key (Borle et al., 1986; Hammami et al., 2009; Loukin et al., 2009; Hoffmann et al., 2014). Changes in cell volume reflect changes in water content, so that anything that affects the flux of water will affect cell volume (Heo et al., 2012; Maneshi et al., 2014). If volume regulation is treated as an equilibrium process, the rates of water transport will not affect the final volume, only the relaxation rates. However, if cell volume regulation were a steady-state process with continuous fluxes, the relative permeabilities to solutes and water would be major factors in setting the cell volume. Aquaporins clearly play a role in volume regulation (Sajja et al., 2014), but they can have no effect on an equilibrium cell volume, as aquaporins are only enzymes that transport water and hence cannot alter the end-state energies. However, if cell volume were a steady-state process, aquaporins could readily affect cell volume. (For the mechanotransduction fans, there is a suggestion that aquaporins are sensitive to membrane tension; Kim et al., 2014.)

Ion fluxes can modify cytoskeletal stresses by biochemical interactions with the structural proteins (notably by

altering calcium levels). The ions could move water by allosterically "squeezing" or "relaxing" the cytoskeleton (Zou et al., 2013). Stresses may also change the local charge density on the proteins and thereby affect the Donnan potentials (Herant et al., 2003), as it is known that mechanical stress can expose or conceal cryptic sites in proteins (Johnson et al., 2007). Thus, the effect of ion fluxes on poroelasticity may involve catalytic rather than osmotic amounts of material. As a reminder of the potential role of ion channels in cell volume regulation, recall that cell volume cannot be regulated by an electrogenic transport of ions. The cytoplasm is electroneutral, and the resting membrane potential is established by an excess of negative ions of only approximately one part in 10^5, far below osmotic significance.

In summary, our long tradition of treating cell volume regulation as the physical chemistry of charged polymers contained in a semipermeable bag (Odijk, 1979) ignores the free energy of the mechanical stresses of a cross-linked cytoskeleton found in most cells (Nieto et al., 2004; Charras et al., 2009). Although we don't yet have a simple probe to measure those poroelastic energies, the data are striking, and for the quantitatively inclined, analytic poroelastic models are available (Cheng et al., 1991; Charras et al., 2009; Taber et al., 2011) as is computational software (COMSOL; Comsol, Inc., or Abaqus; Dassault Systemes). The Appendix provides an example of how to incorporate poroelasticity into the analysis of cell volume.

APPENDIX

An equilibrium analysis of cytoskeletal poroelasticity and membrane stress

Let's think of a cell as a spherical poroelastic cytoskeletal core (a fluid-infiltrated, elastically deformable sponge-like network) confined by an elastic membrane. When water infiltrates the cell as a result of osmosis or other factors, the cytoskeleton swells and the membrane tension increases until the cell reaches a state of mechanical equilibrium. We want to know how much elastic energy is stored in the cytoskeleton compared with that in the membrane. We will restrict ourselves to small deformations so that we can use a linearized theory of poroelasticity. The theory of poroelasticity has its origins in Biot's paper (Biot, 1941), and our approach follows the treatment of Rice and Cleary (Rice and Cleary, 1976; Rice, 1998; Wang, 2000a).

The poroelastic cytoskeleton

The cell is spherical and subjected to small radial deformations. The deformation of the cytoskeleton is specified by two values: (1) the volumetric strain, i.e., the fractional change in volume of the elastic network as a whole, ε_v; and (2) the porosity change, i.e., the fractional change in the volume of pore spaces in the network, ζ. Just like the stress in a spring, there are forces associated with the deformation of the cytoskeleton (ε_v, ζ): (a) the volumetric stress, σ, the force per unit area in the radial direction; and (b) the excess-pore pressure, p, the hydrostatic pressure in the pore spaces. The correspondence of (σ, p) with (ε_v, ζ) can also be thought of in terms of the change in free energy per unit volume, $\sigma d\varepsilon_v - p d\zeta$, much like the familiar $-p dV$ term for gases.

Again, as the force in a spring is related to its deformation by Hooke's law through a spring constant ($F = kx$), (σ, p) are related to (ε_v, ζ) through constitutive equations involving material constants. For our sponge-like poroelastic medium, there are three material constants:

(1) The drained bulk modulus, K: This is the rate of change of volumetric stress σ with volumetric strain ε_v when fluid is allowed to drain freely, i.e., with no change in excess pore pressure p. We can think of this as the stiffness felt when squeezing a sponge slowly, while water is allowed to freely escape.

(2) The undrained bulk modulus, K_u: This is the stiffness when fluid drainage is completely prevented. We can treat this as infinity because we treat water as incompressible.

(3) The Biot–Willis coefficient, α: The volume of the sponge network can be increased either by increasing the radial stress σ, or by increasing the fluid pressure p in the pore spaces (or both). α is the ratio of the stress σ to the pressure p that causes the same increase in volume.

With these material constants, the constitutive equations of poroelasticity, paralleling Hooke's law, can be written as (Rice, 1998):

$$\begin{aligned} \sigma &= K_u \varepsilon_v - \frac{K_u - K}{\alpha}\zeta \\ p &= -\frac{K_u - K}{\alpha}\varepsilon_v + \frac{K_u - K}{\alpha^2}\zeta. \end{aligned} \tag{1}$$

The cell membrane (cortex)

We represent the cell cortex as a homogenous isotropic elastic membrane. The deformation of the cortex is given by the membrane area strain, ε_m, or $\Delta A/A$, where A refers to the membrane area. The corresponding conjugate force–like quantity is the tension T_m (so that $T_m d\varepsilon_m$ is the change in free energy per unit area of the membrane). Again, similar to Hooke's law,

$$T_m = k_m \varepsilon_m. \tag{2}$$

Putting the cytoskeleton and cortex together

When we put the cytoskeleton and cortex together, (a) the forces in the two must be in equilibrium, i.e., the stress in the membrane must equilibrate with the cytoskeletal volumetric stress acting on the inner surface of the membrane; and (b) the deformations of the two must be compatible, i.e., the membrane must expand to accommodate the volume change of the poroelastic cytoskeleton, which is compressed by tension in the membrane. These two conditions give us these equations:

$$T_m = -\frac{\sigma r}{2};\ \varepsilon_m = \frac{\varepsilon_v}{3}. \tag{3}$$

We can recognize that the first of these equations is Laplace's law for spherical pressure vessels. Substituting Eqs. 2 and 3 in 1, we get

$$\varepsilon_v = \frac{\alpha}{\bar{K}} p;\quad \zeta = \left(\frac{\alpha^2}{\bar{K}} + \frac{\alpha^2}{K_u - K}\right) p;\quad \varepsilon_m = \frac{\alpha}{3\bar{K}} p, \tag{4}$$

where we have defined $\bar{K} = K + \frac{2k_m}{3r}$.

Elastic energies stored in the cytoskeleton and the cortex

Analogous to how the elastic energy stored in a stretched spring as $1/2kx^2$, the elastic energy per unit volume in the cytoskeleton is

$$\frac{1}{2}\left(K_u \varepsilon_v^2 + \frac{K_u - K}{\alpha^2}\zeta^2 - 2\frac{K_u - K}{\alpha}\varepsilon_v \zeta\right),$$

and per unit area in the membrane it is $1/2\,k_m\left(2\varepsilon_m^2\right)$ (because the strain ε_m in the membrane is the same in the two mutually perpendicular tangential directions). Thus, if r is the radius of the cell, then the total elastic energies stored in the cytoplasm and the cell cortex are, respectively:

$$\begin{aligned}
\Psi_c &= \frac{4}{3}\pi r^3 \frac{1}{2}\left(K_u \varepsilon_v^2 + \frac{K_u - K}{\alpha^2}\zeta^2 - 2\frac{K_u - K}{\alpha}\varepsilon_v \zeta\right) \\
&= \frac{4}{3}\pi r^3 \frac{\alpha^2}{2}\left(\frac{K}{\bar{K}^2} + \frac{1}{K_u - K}\right) p^2 \\
\Psi_m &= 4\pi r^2 k_m \varepsilon_m^2 = 4\pi r^2 \frac{\alpha^2 k_m}{9\bar{K}^2} p^2.
\end{aligned} \tag{5}$$

Analysis with representative parameters

Taking water as incompressible and the mean bulk modulus of components of the cytoskeleton as K_s, the poroelastic material constants are given by (Rice, 1998),

$$\alpha = 1 - \frac{K}{K_s};\quad K_u = K + \frac{\alpha^2 K_s}{\alpha - n}, \tag{6}$$

where n is the porosity in the reference configuration. If K_s is very large, then α approaches 1, and K_n is very large as well. For a given equilibrium pore pressure, the ratio of elastic energies in the cytoskeleton to that in the cell membrane, Ψ_c/Ψ_m, is of the order of magnitude,

$$\frac{\Psi_c}{\Psi_m} \approx \frac{Kr}{k_m}. \tag{7}$$

Considering the following representative values of the material constants—(a) drained bulk modulus, $K \approx$ 100–1,000 Pa reported in Moeendarbary et al. (2013); (b) $r = 10$ µm; and (c) $k_m = 6 \times 10^{-6}$ N/m reported for the flaccid red cell membrane in Mohandas and Evans (1994)—the ratio of elastic energy in the cytoplasm to that in the cell membrane is ∼500, so that most of the osmotic energy is stored in the cytoplasm. For a volume change of 10 µm^3 ($\varepsilon_v = 0.02$), the change in pore pressure from Eq. 4 is 12Pa ∼10^{-4} atm ∼1.5×10^{-4} psi.

This Perspectives series includes articles by Andersen, Wood, and Haswell and Verslues.

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