

Control of hydrostatic pressure and osmotic stress in 3D cell culture for mechanobiological studies

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ABSTRACT

Hydrostatic pressure (HP) and osmotic stress (OS) play an important role in various biological processes, such as cell proliferation and differentiation. In contrast to canonical mechanical signals transmitted through the anchoring points of the cells with the extracellular matrix, the physical and molecular mechanisms that transduce HP and OS into cellular functions remain elusive. Three-dimensional cell cultures show great promise to replicate physiologically relevant signals in well-defined host bioreactors with the goal of shedding light on hidden aspects of the mechanobiology of HP and OS. This review starts by introducing prevalent mechanisms for the generation of HP and OS signals in biological tissues that are subject to pathophysiological mechanical loading. We then revisit various mechanisms in the mechanotransduction of HP and OS, and describe the current state of the art in bioreactors and biomaterials for the control of the corresponding physical signals.

1. Introduction

Physical forces play an instrumental role in the pathophysiological function of biological tissues from the early stages of development to homeostasis and disease progression [1–6]. To understand the effects of physical forces on cell behavior, bioreactors have been used to recapitulate specific mechanical cues outside of the body with the aim of unraveling their role in cell mechanobiology [7–12]. Bioreactors with finely tuned control of the state of deformation and its dynamics have also been utilized successfully to reveal the effects of complex stretch loading profiles in the mechanobiology of biological tissues in vitro [13–22]. These early bioreactors already pointed out the need to design in vitro systems that do not substantially alter the cellular phenotype in a way that would confound the outcome of mechanobiological studies. Therefore, new biomaterials and bioreactors must be specifically designed in order to reproduce select mechanical and biochemical features of the tissue environment in vitro.

Hydrostatic pressure (HP) and osmotic stress (OS), which are the central topics of this review, define a unique class of pathophysiological

cues [23–25] whose time-dependent effects on cell function remain poorly understood. For this reason, this review is dedicated to the state-of-the-art and recent advances that emulate representative signals of the tissue environment in vitro. Analyses of the interplay between structural composition, mechanical properties, and mechanical loading of soft biological tissues have provided insight on key pathophysiological parameters that should be considered while exploring the cell mechanobiology of HP and OS [26].

The biophysical and biochemical conditions that cells are exposed to in living tissues are complex and they are strongly influenced by the extracellular matrix (ECM) [27]. This complexity is further enhanced when tissues are subjected to external mechanical stimuli since the ECM can translate them into changes of tissue morphology, strains, and also variations in HP and OS [28]. These changes in HP and OS are intrinsic to the microstructure of the tissues: In most load-bearing tissues, such as cartilage, intervertebral disk, and skin, the ECM consists of a network of collagen fibers embedded in a charged, hydrated matrix of proteoglycan-glycosaminoglycan aggregates and hyaluronic acid [29–31]. These negatively charged macromolecules maintain

Abbreviations: HP, Hydrostatic pressure; OS, Osmotic stress; GAGs, Glycosaminoglycans; MSCs, Mesenchymal stem cells.

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physiological levels of hydration (typically 70–80 % of tissue wet weight) by generating osmotic pressure, and they balance the concentration of dissolved ions (e.g., Na^+ , Cl^-) to satisfy chemical equilibrium and electroneutrality [32].

As rationalized by various mathematical models [32–35], several ECM constituents and their corresponding interactions have been shown to cooperatively determine the load-bearing function in biological tissues. For example, typical *in vivo* compressive loads acting on articular cartilage ($\sim 3\text{--}18$ MPa, 0.5–1 Hz) [36] or intervertebral disk ($\sim 0.1\text{--}3$ MPa, 0.1–1 Hz) [37] are predominantly supported by the pressure of the interstitial fluid [38]. This fluid pressure is generated by the load itself and transiently maintained by the relatively low hydraulic permeability ($\sim 10^{-4}\text{--}10^{-3}$ mm⁴ N⁻¹ s⁻¹) of the dense collagen-proteoglycan matrix [38,39].

Together with the hydrostatic pressure, the osmotic pressure generated by the proteoglycan-glycosaminoglycan aggregates sets the chemical potential of the tissue fluid. Differences in the interstitial fluid pressure of the tissue generate gradients in the chemical potential which in turn drives fluid flow. This mechanism causes local changes in water content and thus tissue volume. The latter alters the concentration of fixed and mobile solutes in the tissue, and thereby changes the osmotic pressure within the ground matrix. The chemomechanical properties of the tissue therefore establish a link between variations in HP and OS on the one hand, and local tissue deformations on the other [28,34,40]. At equilibrium conditions, fluid flow and volume change cease, equalizing the residual HP with osmotic pressure, Fig. 1a. Importantly, the effect of mechanical tissue loading on HP and OS is different from a direct change in fluid pressure, as might be expected when varying the environmental pressure (such as in high altitudes or deep water). In fact, the latter type of pressure variation is transferred directly to the cell cytosol without causing significant tissue or cell deformation, see Fig. 1b.

While the importance of multiphase tissue composition has long been appreciated in cartilaginous tissues under compressive loading [33,41], it was recently demonstrated that several connective tissue membranes (e.g., skin, amnion, organ capsules) also respond to tensile loading through chemomechanical coupling [28,42]. The seemingly contradictory coupling between tensile load, pressurization of the interstitial fluid, and subsequent fluid loss was explained by the nonlinear response of the collagen fiber network which reorient and compact under tension [28]. These findings imply that the coupling between applied loads and variations in OS and HP are relevant for a

much wider class of tissues than the typical, compression bearing candidates of the musculoskeletal system. In addition to modulating the levels of OS in the interstitial fluid, the osmotically active macromolecules within the ground matrix enable water retention, limiting fluid loss during mechanical loading [28,40]. Higher density of fixed charges leads to stronger hydration and increased tension of the collagen fibers [43,44] along with larger pressure of the interstitial fluid [43]. An increase of osmolytes in blood plasma however decreases chemical potential and exposes cells to higher osmolarity, tissue dehydration, and consequently lower HP of the interstitial fluid, see Fig. 1c.

These considerations provide a glimpse as to how physiological or pathological mechanical loads can induce local changes in HP and OS, individually or combined, of the extracellular fluid *in vivo*. These interstitial fluid related signals might complement canonical cues of integrin-mediated mechanotransduction, i.e. those due to the interaction of the focal adhesions of the cells with the network of collagen fibers, which lead to a direct transmission of matrix deformation to cells and to changes of cell-perceived stiffness [45]. It remains largely open to date how and to which extent these canonical mechanisms interact with the mechanotransduction of HP and OS. However, cell culture studies on the effects of HP under unconfined and static compressive loads (0–3 MPa for 12 h) [46], and OS under osmolyte addition (sucrose, 250–450 mOsm for 4–24 h) [47] clearly demonstrated that fluid associated cues contribute to the activation of different cell responses.

In this review, we revisit recent findings on the mechanisms that drive the mechanobiology of HP and OS. We provide insights into methods for the measurement of osmolarity and HP in biological tissues, which is critical to elucidate their role during pathophysiological mechanical loadings. Key for advancing this state of the art, we analyze the capacity of existing cell culture systems to replicate physiological signals of HP and OS, highlight their limitations, and suggest future developments.

2. Mechanobiology of osmotic stress

Mechanical loads exerted on biological tissues drive the redistribution of soluble and ECM components, establishing variations or gradients in chemical potential that can activate the mechanotransduction of OS for several type of cells [48–51]. The mechanosensing of OS entails several cellular components and processes, including the cytoplasmic membrane [52–54], the organization of the cell nucleus [55,56], and the

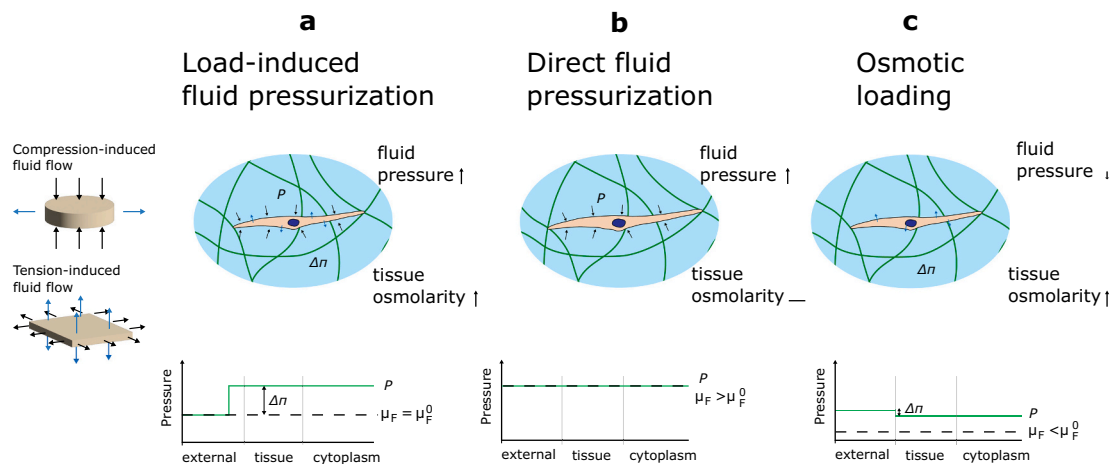


Fig. 1. Biophysics of hydrostatic and osmotic pressure at the cell and tissue scale. The difference $\mu_F = p - \pi$ defines the chemical potential in equilibrium. (a) Typical compression and tensile loads *in vivo* cause tissue deformation and fluid pressurization, which increases the interstitial fluid pressure p and the interstitial osmotic pressure π . These conditions lead to pressurization of the cytosol and flow exchange across the cell membrane. (b) Mechanical pressurization of the external fluid is transferred directly to the cell cytosol without causing tissue or cell deformation. (c) Osmotic stress by addition of osmolytes that are not permeant to cell membrane can cause loss of cell volume and reduction to fluid pressurization. The above representation assumes that the stiffness of the cell membrane can be neglected so that the hydrostatic pressure of the fluid within the cell is identical to the one outside the cell.

dynamic remodeling of the ECM [57]. The disruption of this versatile osmoregulatory apparatus can impair cell volume control, leading to various pathologies such as inflammation and edema [58–60].

In addition, differences of OS on the cell membrane are often compensated by volume changes which take several minutes to adjust the cytoplasmic concentrations of various solutes [61]. In this case, a “pump and leak” mechanism controls volume changes through the transport of K^+ , Cl^- and Na^+ ions, organic osmolytes, and water to restore the initial cell volume [61–63]. These OS-induced changes in cell volume result in the rearrangement of cytoplasmic and membrane molecules, which as a consequence become driving factors for several cell processes, such as migration, survival, and proliferation [61].

The prominent role of cell volume sparked the pursuit of more sensitive biophysical features to track the corresponding changes. Several mechanisms have been proposed thus far to as indicators for the change of cell volume, including membrane stretching, deformation of the cytoskeleton, and molecular crowding [64]. Changes in cell volume can only be accomplished by stretching the cell membrane and deforming connected intracellular structures. Therefore, volume changes in response to hypotonic stimuli are completed within several minutes (~10 min) and are shown to activate different parts of the mechanosensory apparatus that are responsible for the detection of cellular strains and forces, such as the polymerization of the actin network [65–67]. More specific to volumetric deformations are changes in the packing density of the lipid bilayer [53,68] within <1 millisecond [69] and the activation of mechanosensitive membrane proteins, such as the volume-regulated anion channels (VRAC) within seconds to minutes after volume change [61,70].

The reduction of cell volume in tens of minutes can cause equally important changes in the concentration of cytoskeletal proteins, leading to crowded molecular states and events of phase separation [71–74]. In this case, the dissociation kinetics of molecular aggregates establish physical checkpoints that not only alter the mechanical properties of the cells [54,75–78] but also influence the transmission of mechano-transduction signals in response to OS [56,79].

2.1. Methods for the measurement of tissue osmolarity

As described in the previous section, cells sense the external osmotic pressure and potentially respond to match the osmolarity of their cytosol with that of the extracellular fluid. Thus, the quantitative assessment of osmolarity, as regulated by the production of osmolytes and the flux of interstitial fluids [80], is required to understand the mechanobiology of OS in biological tissues. Compatible osmolytes include small molecular

weight sugar alcohols (e.g., sorbitol or mannitol), methylamines (e.g., betaine), and neutral free amino acids and amino acid derivatives (e.g., taurine) [81]. Perturbing osmolytes, which can cause hyperosmotic stress and directly influence homeostasis, include salts, urea, and sugars (e.g., glucose, fructose). Perturbing osmolytes do not generally permeate the cell membrane and are often used to induce short-term osmotic stresses, because cells slowly compensate their effects via the synthesis of organic osmolytes [80]. Similarly, polymeric osmolytes of natural (e.g., dextran) or synthetic origin (e.g., poly(ethylene glycol), PEG) with tunable molecular weight could be used to control the applied osmotic load in different culture systems [57,82,83].

Measurements of tissue osmolarity assess the presence of osmolytes in the biological environment based on the colligative properties of the solution (e.g., boiling point, freezing point) as a function of the osmolyte's concentration [84,85]. Membrane osmometers consist of one chamber with a pure solvent and another with a test solution, separated by a semipermeable membrane (Fig. 2a). To restore osmotic equilibrium, solvent flows from the side of low to that of high osmolyte concentration, causing a measurable change in HP that relates to the osmolarity of the solution (van't Hoff law). Although a well-known technique, membrane osmometry is time consuming and faces the hassle of fabricating membranes that are selectively permeable to specific solutes [86].

As alternative approaches, freezing point and vapor pressure osmometers have been used for the study of biological samples [87,88]. When a solute is added, the chemical potential of the solvent decreases, leading to higher boiling or lower freezing temperatures, respectively. A freezing point osmometer probes the drop in freezing temperature to determine the osmolarity of the solution. In this case, a liquid sample containing a stirring wire is immersed in a bath that is cooled below freezing point (Fig. 2b). Vibration of the wire induces crystallization, releasing thermal energy and elevating the temperature to a plateau value [86]. This thermal process defines a freezing point depression that is proportional to the osmolarity of the solution [89]. This technique could also be applied to measure the osmolarity of the interstitial fluid within biological tissues. However, the relationship between the freezing point and the osmolarity assumes ideal behavior, which may not be displayed by biological samples composed of multiple osmolytes. The use of this technique on biological tissues is also complicated by the effects of sample preparation, e.g. homogenization and evaporation [89–91], which might lead to differences in the amount of solvent and solute with respect to the *in vivo* conditions. On the contrary, the non-destructive technique of vapor pressure osmometry, which exploits the shift of equilibrium between the liquid and vapor phases, is more

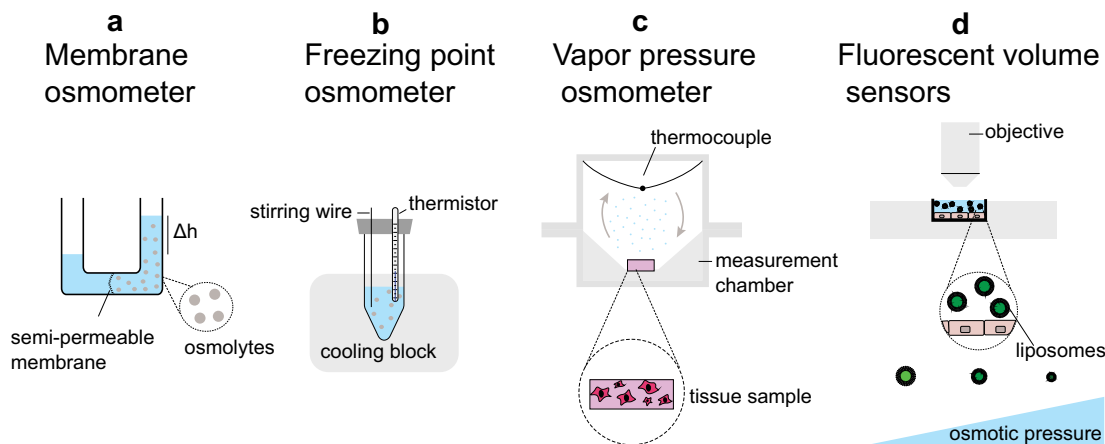


Fig. 2. Different methods for the measurement of osmolarity in biological samples. (a) Membrane and (b) freezing point osmometers measure the osmolarity of a solution containing osmolytes in the liquid phase. In addition to this capability, (c) vapor pressure osmometers can also assess the osmolarity of solid samples incubated in the corresponding chamber. (d) Injectable fluorescent volume sensors such as liposomes loaded with volume-sensitive fluorescent dyes (FRET pairs or self-quenching dye) can potentially measure osmolarity *in vitro* and *in vivo*.

compatible for assessing osmolarity in solid tissues [89]. Vapor pressure osmometers contain a closed chamber for the test sample and a thermocouple hygrometer to detect the characteristic dew point (Fig. 2c) [92]. However, samples with volatile organic compounds violate the assumption of a purely aqueous phase, making them ineligible for characterization with this method [89].

The handling of solid tissues for the measurement of osmolarity often involves sample extraction by means of ultracentrifugation or cutting and snap-freezing in liquid nitrogen [93,94]. Although debated, the latter method has obtained more accurate measurements of osmolarity, as it avoids side contributions from sample destruction [95]. Vapor pressure osmometry on frozen solid samples has also been used in biological tissues (e.g., kidney and skin) with sufficient spatial resolution and good reproducibility [88,94]. Although solid tissues are not subject to homogenization or dilution, the freezing step may cause cell lysis, releasing intracellular material into the sample [87,94]. Despite this progress, none of the described protocols for tissue sampling are suitable for *in vivo* studies. To this end, novel technologies with non-invasive volume-sensitive fluorescent probes may allow for the direct measurement of tissue osmolarity *in vivo* (Fig. 2d). These sensors are composed of liposomes (water-permeable) whose volume correlates with osmotic stress, changing the internal concentration of loaded fluorescent probes. Liposomes, loaded with calcein (self-quenching at high concentrations) and with sulfo-rhodamine (volume-insensitive) as a reference, have been used to detect the local increase (30 %) of the osmolarity in the airway surface liquid of cannulated mice [96]. Calcein in combination with calcium-imaging was also used to track the response of muscle fibers to higher myoplasmic osmolarity in the transverse tubular system of toad and rat animal models [97]. A more recent development was the use of liposomes loaded with Förster Resonance Energy Transfer (FRET) dyes, exploiting the change in FRET efficiency with liposome volume. Simultaneous tracking of liposome volume and FRET efficiency by means of confocal microscopy can allow the mapping of the spatio-temporal changes of osmotic pressure in biological samples [98].

3. Mechanobiology of hydrostatic pressure

It has been reported that HP impacts important biological functions, such as cell death (both apoptosis and necrosis), proliferation, migration, and differentiation in several cell types, engaging a variety of mechanotransduction mechanisms [99–102]. Many lines of evidence show that HP mediates the mobility of Ca^{2+} ions, which in turn play an important role in critical cellular functions, such as the commitment of mesenchymal stem cells (MSC) towards a chondrogenic phenotype [103], the lumen formation in blood vessels [104], and the synaptic plasticity regulated by astrocyte cells [105,106].

HP can also alter the expression of various proteins with an eminent role in cell mechanosensing. For instance, HP boosts the expression of heat-shock proteins which, due to their affinity for the focal adhesion complex [107,108], disrupt the transmission of contractile forces to the ECM [109]. Moreover, HP can influence the expression of integrin proteins that are known for their role in orchestrating force transmission to the ECM [101,105,110,111].

The time history for the application of HP is another important factor that may drive different mechanobiological responses. For instance, acute (1 h) application of HP (100 mmHg) was shown to promote Ca^{2+} -mediated myosin activation, while the same endothelial cell responses subject to chronic (24 h) HP signals (100 mmHg) were dominated by high cortical actin density and loss of barrier function [112]. Both regimes demonstrate cortical stiffening in effect of HP, with concomitant effects on the expression of tubulin and vimentin proteins [112]. The latter cytoplasmic response is consistent with early evidence of chronic exposure to HP (~40–150 mmHg from 1 to 9 days) which triggers the assembly of centrally located stress fibers in cells with elongated morphology, an effect most likely related to high cell density and loss of contact inhibition by reduced expression of VE-cadherins [113,114].

Contradictory findings showed that neither acute (3 h) nor chronic exposure (9 days) to HP (100 mmHg) trigger any major difference on the cytoskeletal organization and the morphology of endothelial cells underwent either slow (30 min) or rapid (~5 s) depressurization protocols [115]. Although not clarified by the latter work, these different cell responses highlight the need for the design of new cell culture models and bioreactors for the systematic investigation of HP mechanobiology.

Additional evidence suggest that acute application of HP induces reversible alterations on cell function. For instance, acute application (1 h) of HP (~1.5–6 mmHg) from the basolateral to the apical side of epithelial cells increased transepithelial conductance through Cl^- transport, following the remodeling of tight junctions and actin cytoskeleton [116]. Still, turning off the imposed HP gradient restored the control function of epithelial cells. In a similar example, the acute application of HP (2 h, 70 mmHg) on floating cancerous cells induced a reversible reduction to their radius through the cross-membrane transport of Na^+ and K^+ ions [117].

Separate studies indicated additional mechanisms of cell mechanotransduction in response to HP. For example, the physical characteristics of the cell membrane, such as shape and fluidity, responded to HP through the activation of molecular signals, like actin filaments and caveola-forming proteins [118–120]. High levels of HP reduce the fluidity of the cell membrane, repressing the expression of lipid desaturase in chondrocyte cells [121]. In a reciprocal manner, high cholesterol composition in cell membrane mitigated the activation of mechanotransduction pathways associated with HP in osteoblast cells, such as the extracellular signal-regulated kinase [122]. Recent findings also highlighted the role of HP in cell mechanotransduction by tracking specific molecular changes of the cell nucleus, such as the remodeling of heterochromatin [123] and the localization of YAP and TAZ proteins [124]. The mechanosensing of HP signals mechanotransduction mechanisms come along Additional evidence shows that pulsatile hydrostatic pressure, replicating the conditions of the lung, initiates an inflammatory response through the mechanically activated ion channel PIEZO1 [125].

3.1. Methods for the measurement of HP *in vivo*

The measurement of HP in biological tissues *in situ* is significantly more challenging than in liquid chambers of 3D cell cultures and bioreactors. At the same time, there is an increasing demand to measure HP in biological tissues for improving new diagnostic and treatment protocols. Adapting similar advancements in bioreactors may provide new evidence for the mechanobiology of HP. For instance, the measurement of blood pressure in cardiovascular tissues is predominantly performed by catheters with an integrated fluid column which can transmit blood pressure to an external transducer. In this case, the geometry and the mechanical compliance of the catheter relative to the contacting tissues are critical features for the acquisition of reliable pressure values [126]. Instead, thin pressure wires overcome several limitations, such as the large size of catheters relative to blood vessels and the inefficient transmission of pressure waves from the tip of the catheter to the transducer [127,128]. Additional modifications to the material properties of the wires shall enable high usability by end users and increased biological safety. The latter is an important technical and clinical challenge associated with foreign body reactions against biomaterials [129]. Thus, the development of novel material methods with the potential to reduce foreign body reactions [130–133] shall facilitate the acquisition of local pressure measurements, untainted by the contributions of adverse tissue reactions. Catheters with miniaturized transducers on their tip have also been used for the measurement of interstitial fluid pressure in tumors [134]. In this case, the catheters were safely guided to the core of the tumor through the lumen of a surgical needle, exhibiting the capacity to measure interstitial fluid pressure of several cm- H_2O .

In a different organ, intraocular pressure defines a key indication for

patients suffering from ocular hypertension or other cases of eye disease. Intraocular pressure is measured using either indentation or applanation tonometry, with Goldmann applanation tonometry constituting the standard method for the measurement of intraocular pressure in vivo [135]. Applanation tonometry is the most reliable method for the measurement of intraocular pressure, as calculated by the force exerted to flatten a fixed area of cornea by means of an illuminated, truncated cone. Despite its widespread clinical use, Goldmann applanation tonometry is sensitive to variations of cornea thickness, the type of contact with the truncated cone, and the handling skills of the end-user. Alternatively, non-contact tonometry (i.e., air-puff tonometry) measures intraocular pressure through a light beam and a sensor that adjust air flow relative to cornea position [136]. Over the years, several techniques for the measurement of intraocular pressure, such as rebound tonometry [137], pneumotonometry [135], and dynamic contour tonometry have been developed. The latter assumes that pressure is equally applied to an enclosed fluid space, and it calculates intraocular pressure without inducing cornea deformation [138]. To fulfill the clinical goal of continuous intraocular pressure monitoring, new methods based on the measurements of electromechanical sensors demonstrate promising results for the efficient read out of intraocular pressure in situ [139].

The measurement of HP is also important in understanding the pathophysiological mechanical loading of tissue joints. For instance, implanted pressure sensors during hemiarthroplasty were used to measure pressure values of up to ~18 MPa at the hip joint [140]. Percutaneous sensors were implanted during arthroscopy to monitor cartilage-on-cartilage pressure [141]. However, the in-situ measurement of HP in tissue joints remains a significant challenge. Within this aim, telemetry systems, powered by inductive coupling or batteries, have successfully measured the forces developed in tissue joints in situ [142].

New strategies for the physical characterization of biological tissues could also provide a solution for the measurement of HP. For instance, microneedle injection has been used to measure the infusion pressure in soft tissues. In this case, microneedles were connected to an external manometer and monitored with live imaging to correlate syringe movement to pressure signals [143]. Specifically, microneedles of control length were inserted at different depths of the skin to inject sterile saline solution with a constant flow rate. Interestingly, microneedles encountered changes in infusion pressure at different depths of the human skin, an effect attributed to the varying organization of the ECM at the different layers of the skin tissue [143]. Separate studies demonstrated the capacity to measure similar back-pressure effects by subcutaneous microneedle injection in vivo [144]. The development of advanced protocols of microneedle injection for optimizing the flow rate, the rheological properties of the solute, and the volume injected are expected to facilitate future studies for the measurement of HP in situ.

4. Conventional and advanced bioreactors for 3D mechanobiological studies

Early studies in the design of bioreactors focused on applications of tissue regeneration [145–151], combining knowledge on the chemical and biological performance of different constructive materials, patterns of mechanical stimulation, and cell characterization tools [8,152–158]. Integrating this basis with recent breakthroughs in tissue fabrication, biophysics, and biomaterials, should enable bottom-up strategies for the design of bioreactors that are well-suited to investigate the mechanobiology of HP and OS. Novel methods in 3D tissue bioprinting [159–163], mechanical characterization of biological tissues [164–169], the formulation of responsive biomaterials with tunable biomechanical and biochemical properties [170–176], and the development of 3D organotypic cell cultures [177–182] highlight a battery of innovations that can be exploited towards the design of advanced materials systems and bioreactors for the control of HP and OS.

4.1. Bioreactors and biomaterials for controlled application of osmotic stress

4.1.1. Regulation of osmotic stress by solute addition

OS is typically studied in vitro by regulating the osmolarity of cell culture media, a condition that can be tuned without the use of dedicated bioreactors. For instance, hypertonicity is often induced by adding solutes in the media, such as sorbitol [183], PEG [184] or sodium chloride (NaCl) [185]. Sorbitol and PEG are considered biologically inert, while NaCl ions permeate the cell membrane with the possibility of activating cell responses that are complementary to OS [186]. Moreover, the molecular weight of solutes provides a mean to obtain control over the distribution of OS in the biological system. For instance, the molecular weight of dextran, whose relationship between concentration and osmotic pressure is well-calibrated [187,188], shall be carefully chosen in order to control the distribution of the pressure load into the tissue. Previous studies used dextran molecules of high and low molecular weight to apply osmotic pressure either onto the outer surface of the multicellular spheroids or onto the single cells within [57,82]. Thus, this strategy harnesses the capacity of osmotic agents to infiltrate in 3D tissues as a function of their molecular size and hydrodynamic radius [83].

On the other hand, hypotonicity is often obtained by adding urea to the media, as indicated by the swelling of red blood cells under iso-osmolar urea solutions [189]. Because the tonicity of cell culture media containing urea solutions is not easily quantifiable, the accurate magnitude of obtained hypotonic stimulus remains obscure. Another method to apply hypotonic conditions is by diluting cell culture media with distilled water [76]. This approach however introduces confounding factors associated with the reduced concentration of supplementary media components, such as glucose and growth factors. The dimensionality of the cell culture [190,191] and the application of mechanical strains [21] define additional bioreactor features that can be tuned to replicate physiologically relevant signals of OS.

4.1.2. Biomaterials for chemomechanical coupling

Active biomaterials [174,192–194] can be used to reproduce the chemomechanical coupling between pathophysiological mechanical loadings and OS. For example, composite biomaterials containing polyelectrolytes and collagen fibers capture the mechanism of osmotic swelling of the extracellular matrix [195], resembling both the composition and multiphasic loading function observed in several biological tissues. The fixed density of charges related to polyelectrolytes establishes gradients of chemical potential that influence the directionality of interstitial fluid flow and concomitantly the effects of OS [196]. Separate studies developed scaffolds with fixed-charges through chondroitin sulfate (CHS)-modified PEG hydrogels [197]. These scaffolds exhibited high ion concentration and enhanced tissue growth under compressive loading in plain culture media (330 mOsm, 1 Hz, 15 % strain for 6 h). Interestingly, chelation of Ca^{2+} signaling inhibited the synthesis of glycosaminoglycans (GAGs) by chondrocyte cells under similar conditions of compressive loading, suggesting a complex mechanism of OS mechanotransduction in the corresponding gels.

3D scaffolds of natural polysaccharides without adhesive ligands offer a biomaterial platform to assess OS mechanotransduction, decoupled from canonical signals mediated by focal adhesions. For instance, ligand-free alginate scaffolds with canine notochordal cells were used to investigate the effects of OS in intervertebral disk differentiation [198]. In this case, hyperosmotic conditions (400 mOsm/L for 1–24 days) in effect of added ionic osmolytes (i.e., NaCl and KCl) promoted the production of negatively charged GAGs that alter the dynamic characteristics of the extracellular matrix. Scaffolds composed of agarose, a different polysaccharide, demonstrated low cell viability and weak differentiation of human adipose-derived stem cells (hASC) under similar hyperosmolarity signals (400–600 mOsm/L) after several days of culture [199].

These biomaterial formulations are deprived of ECM proteins which are known to engage ligand-specific cell adhesion events in biological tissues. Recent studies used PEG-CHS gels with adhesive ligands [200]. In this case, dynamic loading of MSC-seeded gels (1 Hz, 2.5 % amplitude strain for 1 h) promoted chondrogenesis through the activation of osmo-responsive transcription factors. Following the increasing need for bioreactors with organotypic complexity, separate studies explored the mechanotransduction of OS in cell culture systems *ex vivo* [180,201–203]. In a similar example, tissue segments of mouse intervertebral disk were cultured in multi-well plates under continuous or intermittent loads of OS, finely tuned by the added dose of sucrose solutions [204]. Static (14 days), burst (90 min/day for 14 days), and cyclic (10 min on/off for 90 min/day for 14 days) of the same magnitude (500 mOsm/L) were used to emulate the osmolarity conditions experienced during tissue growth, loading from brief daily exercise, and short daily movements, correspondingly. In this case, it was only the static hyperosmotic loading that triggered the expression of osmoregulatory ion channels (i.e., aquaporin), with additional effects on the expression of mechano-regulatory membrane proteins in notochordal cells.

4.1.3. Bioreactors with controlled signals of hydrostatic pressure

Bioreactors with well-defined signals of HP often require a minimal number of parts, including a cell culture model in a pressure chamber, a connection line attached to a pressure source, and a logic unit for the temporal control of pressure. Up to date, many studies focused on the design of bioreactors that replicate the physiological magnitude of HP signals in articular cartilage and intervertebral disk tissues [205–209]. These tissues demonstrate high water content which is sufficiently pressurized under typical compressive loads (0.2–10 MPa) [36,210]. Moreover, many compressive and tensile loads are exerted on biological tissues within a range of dynamic motions performed by the human body (<1 Hz) [211,212]. Despite the availability of results from the extended investigation of HP in 2D cell culture [112–114,123], this review focuses on 3D bioreactors that have been previously developed to model the effects of dynamic HP in the function of biological tissues.

The use of compression systems is a common strategy to impose controllable patterns of fluid pressurization in 3D microtissues *in vitro*. Bioreactors comprised of a hydraulic loading frame and a liquid-filled chamber have effectively been used to apply intermittent loads of HP (0.1, 1, and 10 MPa) on 3D pellets of human MSCs for 14 days (4 h/day) at a frequency of 1 Hz [213]. Similarly, bioreactors comprised of a hydraulic piston and a water-filled pressure chamber have been used to exert pulsatile signals of HP (1 MPa, 1 Hz, 4 h per day) on collagen scaffolds of human bone marrow stromal cells for 10 days [214]. Pursuing a longer period of cell culture, Meyer et al. connected a hydraulic cylinder with an Instron to apply cyclic HP loads (1 h/day, 5 days/week, 1 Hz, 10 MPa) on alginate scaffolds of MSCs from different donors for 42 days [215].

Different bioreactor set-ups controlled HP by applying mechanical loads at the proximal air-liquid interface of the cell culture. Correia et al. used a pressurized air source with a programmable logic controller to transfer time-dependent HP loads of selected magnitude (~MPa) and frequency (0–1 Hz) for 3 weeks (3 h/day, 5 days/week) [216]. The latter compression system featured a stainless-steel vessel and a polypropylene syringe tube to obtain either physiological (1.5–15 MPa) or low (0–0.5 MPa) mechanical loadings for cartilaginous tissues, respectively. Similarly, Reinwald et al. used an Instron device to transmit cyclic signals of HP (0–270 kPa, 1 Hz, 1 h per day for 14 days) on Matrigel scaffolds of skeletal cells seeded at the air-liquid interface of transwell inserts [217].

The complex and dynamic microenvironment of the natural tissues has also inspired the design of new HP bioreactors with additional physiological similarities. For instance, biomaterials scaffolds with select mechanical and biochemical properties have been used to better replicate the HP transmission in the heterogeneous tissue microenvironment. With this objective, Aprile et al. used an interpenetrating hydrogel scaffold (collagen and alginate) with tunable stiffness (5 vs 17

kPa) to establish a 3D culture of MSCs in a bioreactor [218]. In this example, the application of pulsatile HP (2 MPa, 1 Hz, 4 h per day for 7 days) triggered distinct patterns of cell aggregation and chondrogenic marker expression that was mediated by the stiffness of the scaffolds [218]. Using a different strategy, Nazempour et al. focused on the combined effects of HP and shear stress on biological responses [219]. In this case, a stainless-steel bioreactor was connected to both a hydraulic cylinder and a flow channel. Interestingly, the simultaneous application of shear stress (0.02 Pa, 0.5 mL/min) and oscillating HP (4 MPa, 0.5 Hz) on 3D agarose scaffolds of bovine articular chondrocytes led to a higher density and stiffer matrix compared to shear stress alone.

A different line of studies created bioreactors for the controlled application of HP on organotypic tissues *ex vivo*. Wang et al. used a pressure device to apply various loads of HP (0.5–5 MPa, every 30 s for 2 h) on intervertebral disk specimens [124], which were then incubated in cell culture chambers for 30 days. Interestingly, the initial HP shock was sufficient to promote significant differences on the proliferation and viability of the cells in the corresponding tissues. Furthermore, Ingensiep et al. created a multi-well culture chamber of retina tissue segments in connection to a hydrostatic column to apply supraphysiological HP (1.3–12 kPa) in acute steps of 2 h and consequently measure their electrical activity [220]. Building a different bioreactor, Ishikawa et al. connected a pressurized gas tank to a cell culture beaker to replicate HP signals (1.3–9.9 kPa, 24 h) that are linked to retina tissue damage [221].

5. Custom bioreactors for the control of osmotic stress and hydrostatic pressure

3D culture systems have significantly increased our understanding on the mechanobiology of HP and OS [36,57,82,195,209]. These systems have primarily used the mechanical pressurization of the extracellular fluid (Fig. 1b) and the generation of osmotic stress by osmolyte addition (Fig. 1c) to reveal hidden mechanisms of cell mechanotransduction. At the same time, there is a strong incentive to continue developing dedicated bioreactors with new technical capabilities for improving both their biomimetic characteristics and reading capacity [7,8,10,153].

5.1. Bioreactors for *in situ* monitoring

Technical advancements that enable the *in-situ* assessment of tissue function in bioreactors have the potential to generate new evidence in cell mechanotransduction [153]. In this context, we fabricated bioreactors with ad hoc interfaces to capture live cellular responses to the signals of HP and OS (Fig. 3a) [222]. This bioreactor uses a hydrostatic column similar to Ingensiep et al. [220] in combination with a sealed system for controlled HP (~0–20 kPa). In this case, the use of polymer membranes, which are permeable to CO₂, impermeable to liquids, and permissive to the transmission of fluorescent light, defines a critical technical parameter to enable monitoring of fast mechanotransduction responses to HP, such as ion transport through the cell membrane [103–106,112]. Using a similar membrane at the bottom of our bioreactor, we correlated the effects of HP with the Ca²⁺ signaling of human dermal fibroblasts (hDFs) by live cell imaging [222]. Technical modifications enabling *in situ* monitoring may be essential to clarify the effects of short time effects of HP and OS in cell function [113–115].

5.2. Perfused bioreactors

Significant advancements have been made in the control of fluid flow in 3D bioreactors which are primarily driven by tissue engineering applications [146,223,224]. Many lines of evidence show that perfusion influences the mechanotransduction of HP in 3D bioreactors with increased physiological output [225–228] [219]. In this direction, we designed a 3D bioreactor to exert controllable signals of HP (~0–20 kPa) on free-floating scaffolds of hDFs with simultaneous media perfusion

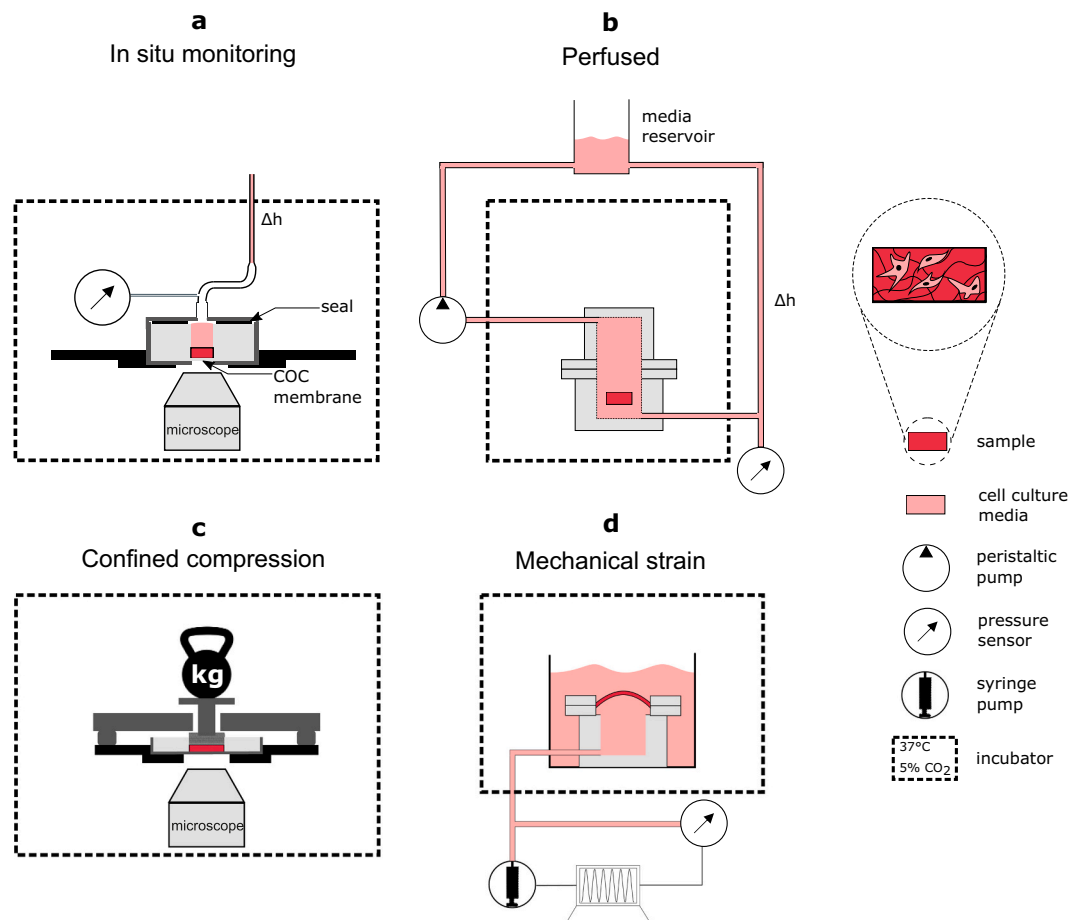


Fig. 3. In-house bioreactors for the control of HP and OS in 3D scaffolds. (a) Bioreactor consisting of a hollow cylindrical polyvinyl chloride (PVC) chamber, sealed at the bottom with a disk made of cyclic olefin copolymer (COC) and at the top with an intermediate rubber element. The COC disk lies between a lid and a hollow support for microscopy access. A water-filled column, connected to a pressure sensor, applies HP on the sample. (b) This bioreactor features a two-part custom-built polyoxymethylene (POM) chamber for the incubation of free-floating 3D hydrogels. The chamber is perfused with media via gas-permeable silicone tubes that are connected to an external media reservoir by means of a peristaltic pump. The pressure sensor is connected to a computer for continuous recording of HP. The vertical position of the external reservoir and the concentration of osmolytes in the media control the magnitude of HP and OS exerted on the free-floating samples. (c) The bioreactor for confined compression consists of a hollow PVC cylinder that contains the 3D scaffold immersed in cell culture media. By means of a known weight, the guided porous filter on top of the scaffold transmits a mechanical load while allowing fluid outflow. Similar to (a), the lower part of this bioreactor permits live monitoring of cell behavior via fluorescent microscopy. (d) This mechanical strain bioreactor can potentially be combined with the above systems (a-c). It contains a 3D scaffold which is circumferentially anchored between two clamps and subject to cyclic mechanical strain by means of a syringe pump [21]. These components are stabilized on a chamber which is connected to the syringe pump and immersed in culture media for constant supply of nutrients.

(Fig. 3b). HP was controlled by adjusting the vertical position of an external media reservoir similar to previous set-ups [114,229]. The use of an external peristaltic pump controls the rate of perfusion by gas-permeable tubing that enables media equilibration, independent of the rate of O_2 and CO_2 adsorption by the media. The bioreactor chamber accommodates multiple free-floating 3D cell-laden scaffolds for long periods (~weeks), and it is adjustable to the use of different osmolytes for the control of OS.

5.3. Confined compression bioreactors

The tissue microenvironment is often subject to multiple compressive and tensile loads that may induce concurrent changes in OS and HP (Fig. 1a) [28,34,40]. In various examples, the unconfined compression of 3D scaffolds has provided strong evidence on the remodeling of the 3D cell microenvironment with charged macromolecules that influence osmotic pressure and chemical potential [43,46,197,200]. As the time and magnitude of the compression, in relation to the mechanical and biochemical properties of the 3D scaffolds, is known to affect the origin of HP and OS [44,230,231], we designed a confined compression

bioreactor to probe the change of osmotic and hydrostatic pressure for controllable mechanical loadings (Fig. 3c) [34]. The confined compression is exerted by a porous filter for fluid outflow as a function of the composition and fluid pressure of the 3D scaffold, as previously suggested [38,39].

5.4. Mechanical strain bioreactors

Bioreactors with the capacity to apply controllable mechanical strain *in vitro* have been extensively used and discussed in the past for tissue engineering and mechanobiological applications [11,13,15,22,232]. These advancements could establish an experimental platform to systematically investigate the mechanobiology of HP and OS under well-controlled dynamic tissue deformation without confounding effects from the change of cell phenotype. To this end, our lab has previously developed a mechanical strain bioreactor that applies cyclic biaxial tension on skin substitutes, leading to highly proliferative cells without phenotypic alterations (Fig. 3d) [21]. This bioreactor demonstrates the use of physical cues to create large and fibrotic-free skin substitutes that could be used to tackle the clinical need of large autologous skin grafts

[233–235]. The incorporation of HP and OS signals in similar mechanical strain bioreactors [7,15,20,236–238] could generate new solutions for tissue engineering and mechanobiological applications.

6. Future perspective for in vitro systems to study hydrostatic pressure and osmotic stress

The design of bioreactors that mimic pathophysiological chemomechanical effects of biological tissues in the natural cell microenvironment will provide a powerful playground to elucidate different aspects of cell mechanobiology related to the physical signals of HP and OS. Advancements in this direction shall entail the formulation of 3D cell culture systems (biomaterials) that replicate the structural features, the mechanical characteristics, and the pathophysiological loadings of different biological tissues. The fabrication of dynamic cell culture systems that concomitantly reproduce physiological tissue deformation in response to external mechanical loading will be an important step for the study of HP and OS in cell mechanobiology. In this direction, biocompatible scaffolds with the capacity to enable physiologically relevant conditions of chemomechanical coupling will be relevant in these investigations. Furthermore, bioreactors with new modules of mass transfer have the potential to control the interstitial fluid flow of various osmolytes that are important in different cases of pathophysiological loadings. Advancing perfused bioreactors [224,239–243] or developing new composite scaffolds with embedded microparticles [244–246] describe two plausible methods to tune the concentration profile of various osmolytes. Finally, the discovery of new physical indicators of the cell microenvironment will be critical to measure the effects of HP and OS. For this purpose, bioreactors integrated with various techniques of in situ characterization [167–169,247–251] shall reveal new physical and biochemical patterns of cell behavior, closely correlated with the role of HP and OS in cell mechanobiology. Beyond fundamental insight, we envision that a deeper understanding of the mechanobiology of HP and OS can lead to advanced therapies. This can potentially be accomplished by discovering new HP and/or OS related protein targets [112,117,123,125] and biophysical patterns [57,82,195,197] related to disease progression and tissue regeneration.

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Declaration of competing interest

The authors declare no conflict of interest.

Data availability

No data was used for the research described in the article.

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