Optical sensing of mechanical pressure based on diffusion measurement in polyacrylamide cell-like barometers

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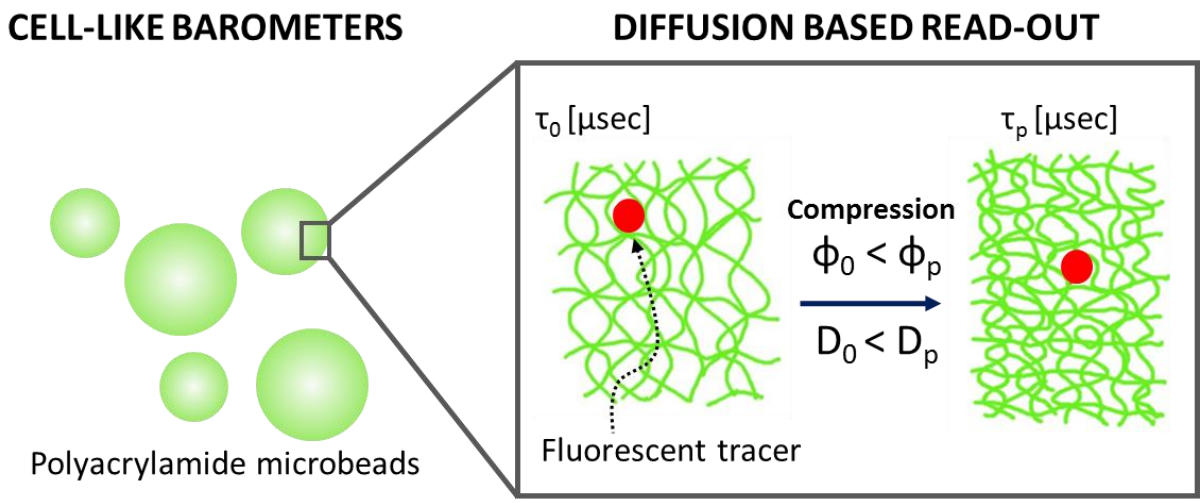
Abstract

Diffusion and transport of small molecules within hydrogel networks are of high interest for i.e. biomedical and pharmaceutical research. In here, using fluorescence correlation spectroscopy (FCS) we experimentally show that the diffusion time in the hydrogel is directly related to the mechanical state (compression or swelling), and thus to the volume fraction of the gel. Following this observation, we develop cell-like barometers in a form of PAA microbeads, which when incorporated in between cells and combined with a diffusion-based optical readout, can serve as the first biosensors to measure the local pressure inside a growing biological tissues. To illustrate the potential of our method, we use multicellular spheroids (MCS) as a tissue model, and we show that the growth-associated tissue stress is lower than 1 kPa, but increases significantly when an external compressive stress is applied.



Diffusion coefficient (D) of a tracer inside hydrogels decreases under compression due to the changes in volume fraction (ϕ). We use this phenomenon to measure tissue stress.

Main section

Cells are constantly exposed to various biochemical and mechanical signals, which together define the state of tissue homeostasis or active processes such as development or morphogenesis. While biochemical signalling plays a major role in the control of cell proliferation and cell death, mechanical stress has proved to regulate how cells move, change their shape and rearrange[1]. Therefore, in order to study complex biological processes it is necessary to have experimental tools that will enable to correlate genetic determinants with mechanical cues[2, 3]. Over the last decades technological improvement made it possible to study genetic determinants within cells. However, research on the role of mechanical cues is still hampered by the lack of direct methods to quantify stresses within tissues. The recently available tool-box of diverse methods applicable to *in vitro* models includes atomic force microscopy, micropipette aspiration, laser ablation (reviewed in ref.[4]), and for *in vivo* models, these methods include for example magnetic resonance[5] and wick-in-needle technique[6]. Recently, an innovative approach has been proposed by Campas *et al* who introduced functionalized oil microdroplets as force transducers to quantify anisotropy of cell-generated forces acting on the droplets within *in vivo* and *in vitro* tissue models[7]. However, this method is incompatible to measure the isotropic component of the stress (tissue pressure) due to oil-droplets incompressibility.

To assess isotropic stress, we introduce a new approach that combines the use of mechanically characterized compressible polyacrylamide microbeads with a direct and rapid optical read-out based on the fluorescence correlation spectroscopy measurement of the diffusion time of small fluorescent tracers. Our technique is based on the hindering of diffusion of a small solute, due to a combination of hydrodynamic and steric effects imposed on the gel. The composition of the gel (ratio of acrylamide to bisacrylamide) has been optimized to be sensitive to physiologically relevant pressures of few kPa. When an external stress is applied to a polyacrylamide (PAA) microbead alone or within the tissue, the induced strain increases the polymer volume fraction, which slows down the diffusion. The corresponding increase in diffusion time due to the gel compression is large enough to be measured by means of FCS. Although the theoretical description is far from being complete, one of the key parameters to account for hindered diffusion in hydrogels is the polymer volume fraction, ϕ[8].

To the best of our knowledge, diffusion-based readout has not been used to date to measure the hydrogel deformation due to the applied stress. Therefore, to demonstrate that a mechanical stress exerted on a PAA gel (ratio of 5% to 0.225% of acrylamide to bisacrylamide (w/w) used throughout the manuscript) effectively has an impact on the diffusion of a small fluorescent dye (sulforhodamine B, hereafter called SRB, hydrodynamic radius 0.5 nm), we placed a steel bead on top of a flat gel (Figure 1a) to locally compress it (Figure 1B) and measure resulting characteristic diffusion time of SRB tracer. To define the compression of the gel, we measured the macroscopic strain δ defined as $(h_0 - h)/h_0$ by

means of confocal microscopy. A plot of the strain is shown in Figure 1C. To correlate the compression with the resultant diffusion time we performed FCS measurements in the gel along a horizontal line below the steel bead. The evolution of the diffusion time of SRB along this line shows that it increases upon the compression (Figure 1C).

To accurately correlate the diffusion time and the mechanical stress, we exerted a well-controlled mechano-osmotic stress (as previously described, ref.[9]) on mechanically homogenous PAA microbeads (Figure 2 and Figure S1) which served as barometers. The evolution of the strain $(V_0 - V)/V_0$ in the PAA gel is measured by image analysis (Figure 2A insert), for pressure ranging from 0 to 10 kPa, and it is presented in Figure 2A. For a given pressure, the strain variation from one bead to another is smaller than 10% (see error bars on Figure 2A).By fitting the strain-stress curve at the origin we determined a bulk modulus K = 20 $\pm$ 1 kPa. For deformations larger than 20%, we observe the well-known[10] deviation of the strain-stress relation from a linear elastic material model.

In parallel, we measured the diffusion time of SRB within the PAA microbeads, as a function of the osmotic pressure. In accordance with experiments which show the evolution of strain as a function of pressure (indentation with a steel bead), the diffusion time increases monotonously with the increasing mechano-osmotic pressure (Figure 2B). This measurable effect has never been reported so far in the literature, although it is well known that diffusion in gels is slower than in free solution. For the formulations of the PAA gel used in this study the diffusion time in the gel at zero osmotic pressure is twice slower than in PBS, τ_W. We define hereafter a normalized diffusion time $\tau^*(P) = \tau(P)/\tau_w$. The data plotted in Figure 2B indicate that τ^* increases linearly with the pressure up to 10 kPa, with a slope of $6.7\pm0.5\times10^{-2}$ kPa^{-1}. Considering that the standard deviation is 7×10^{-2} kPa^{-1}, we estimate that we are sensitive to an absolute stress of about 1 kPa. It is important to note that the typical error of the diffusion time measurement in PBS solution is in the range of 0.1 µsec (S.D.), whereas it increases to 1.5 µsec (S.D.) when measured within the PAA beads. The difference, which defines the sensitivity of the pressure measurements, comes from the possible interactions of the fluorophore and the PAA and also from small inhomogeneities between beads. Calibration curves such as the ones in Figure 2B can be obtained for other gel formulations or different solvents and used for measurement of isotropic mechanical stress in living-materials (see Figure S2 for additional examples of calibration curves).

Although numerous competing models have been proposed to describe solute diffusion within hydrogels, PAA are better described using a hydrodynamic-scaling approach[11]. In this model the polymer chains of the gel enhance the frictional drag on the solute by slowing down the fluid. As a consequence the SRB diffusion constant depends on the volume fraction of the polymer in the gel, ϕ. To ascertain that a unified picture of solute diffusion in our microbeads can be based on the volume fraction, we compared the diffusion time of SRB within gels of different volume fractions ϕ obtained by three different means:

i) flat PAA gels of eight different formulations (from soft to stiff), to vary the volume fraction at equilibrium under no pressure conditions; ii) soft flat PAA gel indented by the steel bead and iii), soft PAA gel beads submitted to osmotic pressures. The volume fractions ϕ_0 of the gels under zero pressure was measured by weighing both the hydrated gel at the equilibrium (m_{wet}), and the remaining weight (m_{dry}), after gels were dried for 3 days. Therefore, the volume fraction was computed as $\phi_0 = m_{dry}/m_{wet}$ (we neglected the difference in density between polymer and water) (Figure S3). The volume fraction under mechanical (steal bead) and osmotic pressure was calculated with $\phi = \phi_0 V_0/V$. Our results indicate that the diffusion depends only on the volume fraction, whatever means has been used to reach this state (Figure 3).

To illustrate a potential application of cell-like barometers, we employed them to define the pre-constraints within the multicellular spheroids. PAA microbeads functionalized with the fibronectin were incorporated in between cells of MCS to sense locally the tissue pressure. We let the spheroids adhere to the surface (Figure 4A and Figure 4B) to limit optical aberrations induced by the rough spheroid surface. We measured the characteristic diffusion time τ^* for beads inserted in the flattened MCS (τ^*= 1.52 ± 0.05, N=16; ±S.E.M) and control beads (τ^*= 1.48 ± 0.06, N=8; ±S.E.M) remaining in the medium during the experiment (Figure 4C). We found a non-significant (p=0.1336) difference between the initial tissue pressure in MCS and the control. As described before, with the sensitivity of 1 kPa of our cell-like barometers our results indicate that the pre-stress inside the growing spheroid remains relatively small. On the contrary, inserted PAA microbeads are sensitive when an external pressure of 5kPa is applied to the spheroid, and the normalized diffusion time ($\tau' = \tau(P)/\tau(0)$, where $\tau(0)$ diffusion time in the bead without any pressure, and $\tau(P)$ is diffusion time in bead under pressure) increases to τ' = 1.25±0.03 (±S.E.M).

Cell-like barometers with a direct optical readout present important advantages over previously published method[12], where propagation of stress within spheroids has been quantified. Direct optical readout based on FCS measurement allows determining absolute local stress at any moment of the tissue development, contrary to image based analysis, which was used to determine changes in local stress within spheroids upon externally applied compression. The limitations of image based analysis are associated with the incomplete information about the initial state of the sensors, and are experimentally difficult to overcome. The direct optical read-out proposed here overcomes these limitations and allows monitoring both local pressure changes that arise from the tissue growth and changes associated with the externally applied stress.

Conclusions

We experimentally demonstrated that the diffusion time of small fluorescent tracers in mechanically compressed PAA gels depends on the volume fraction of the gel. Our experiments show also that such direct optical read-out, based on a diffusion measurement, enables to estimate the absolute value of the tissue pressure within the presented here 3D model system without need of any image analysis or reconstruction. Moreover, we showed that cell-like barometers can be also used to detect the changes in the tissue pressure when external mechanical stress was applied. In future, we anticipate a growing interest to use of such hydrogel based cell-like biosensors to probe mechanics in more relevant systems i.e. organoid *in vitro* models or *in vivo* animal systems to reveal the role of mechanics in development, morphogenesis and disease.

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Competing financial interests

The authors declare no competing financial interests

Acknowledgments

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Figures

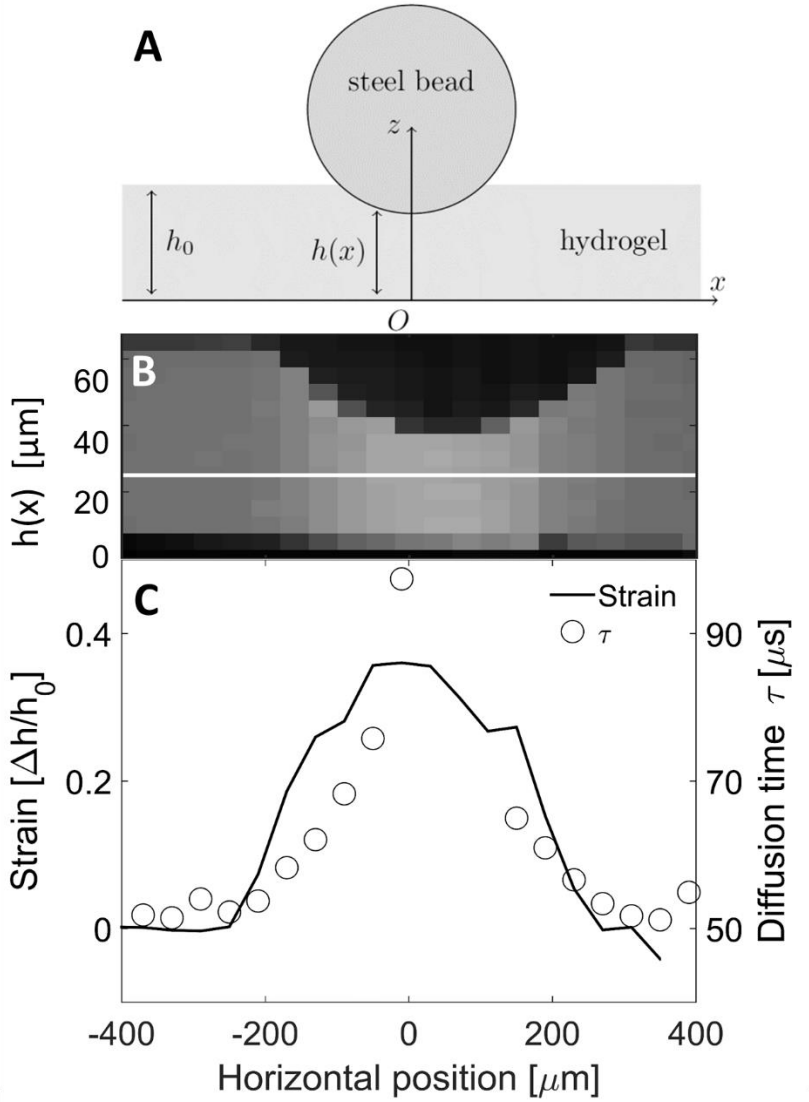

Figure 1 Local and mechanical compression of PAA gel. (**a**) Schematic representation of the experiment where a steel bead (2.38 mm diam.) is indenting a flat PAA gel. (**b**) Image of the SRB fluorescence in a vertical plane (aspect ratio is different from 1). The gel thickness can be measured by detecting the gel interfaces. The white line depicts the positions along which the FCS measurements are performed. (**c**) Plot of the strain $\delta = (h_0 - h)/h_0$ and of the diffusion time measured at z = 25 µm in the gel below the steel bead. Because PAA gels are chemically attached to the surface there is no extension in XY upon compression. As consequence $(h_0 - h)/h_0 = (V_0\text{-}V)/V_0$.

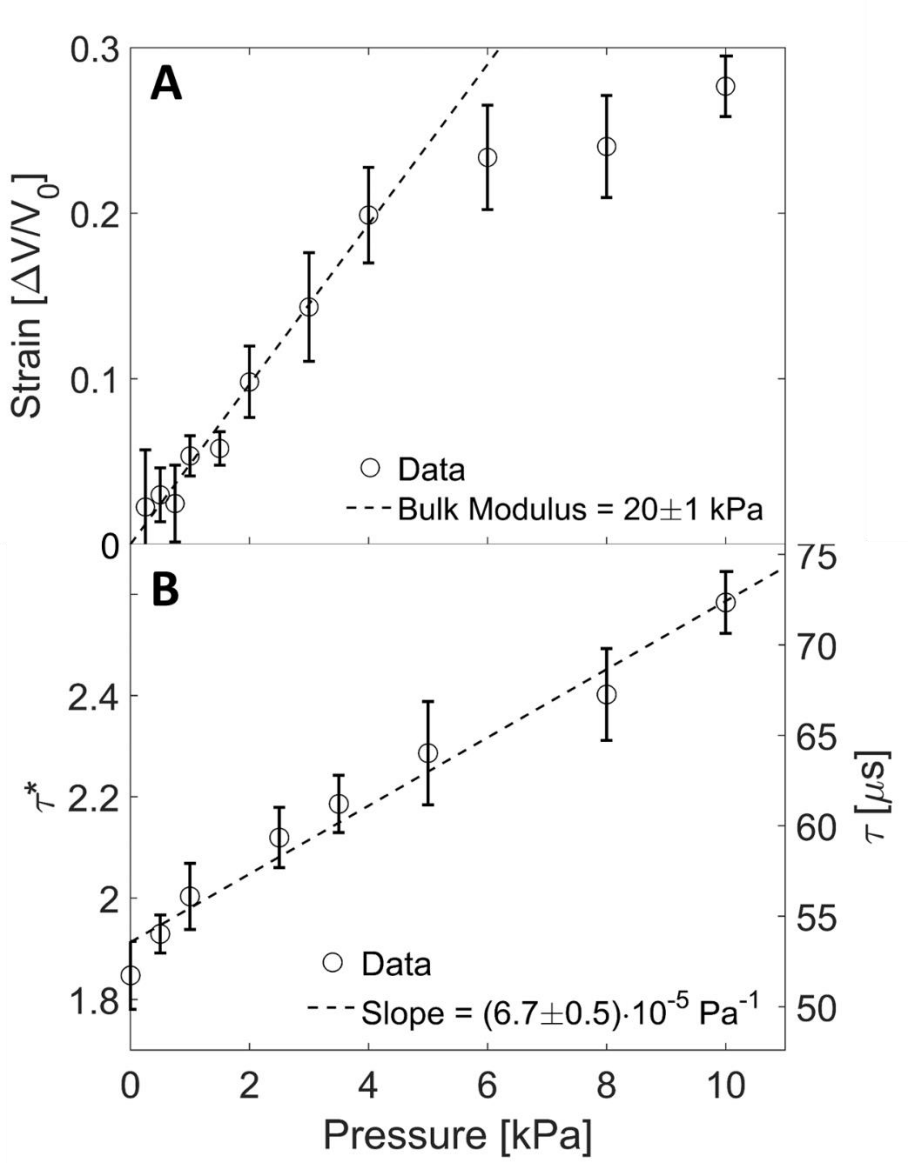

Figure 2 Calibration of the cell-like barometers. (**a**) Evolution of the strain δ for PAA beads as a function of the applied mechano-osmotic stress. The straight line depicts the compression of a linear elastic material of bulk modulus K = 20 ± 1 kPa in the limit of small compression. Error bars are standard deviations. Insert presents fluorescent images of bead before (P=0kPa) and after compression (P=5kPa) (FITC). Scale bar 25 µm. (**b**) Normalised diffusion time measured in PAA microbeads under different osmotic pressures. Error bars are the standard deviations.

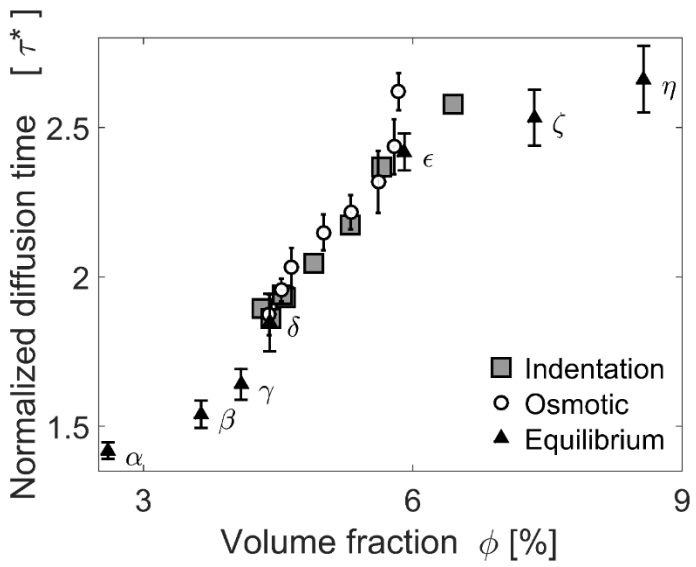

Figure 3: Normalised diffusion time τ* of SRB as a function of the volume fraction ϕ of PAA. The PAA volume fraction has been varied in three different ways: (i) with a mechanical compression, by imposing a mechanical indentation, as shown in Figure 1 (filled squares); (ii) via an osmotic compression, as reported in Figure 2 (empty circles); (iii) using different formulations to vary the mass concentration of PAA (filled triangles). In the latter case, the formulations are respectively: α) 3% Acrylamide - 0.225% Bis-acrylamide, β) 4% A – 0.225% B, γ) 5% A – 0.15% B, δ) 5% A – 0.225% B, ε) 6.5% A 0.225% B, ζ) 8% A – 0.225% B, η) 10% A, 0.225% B.

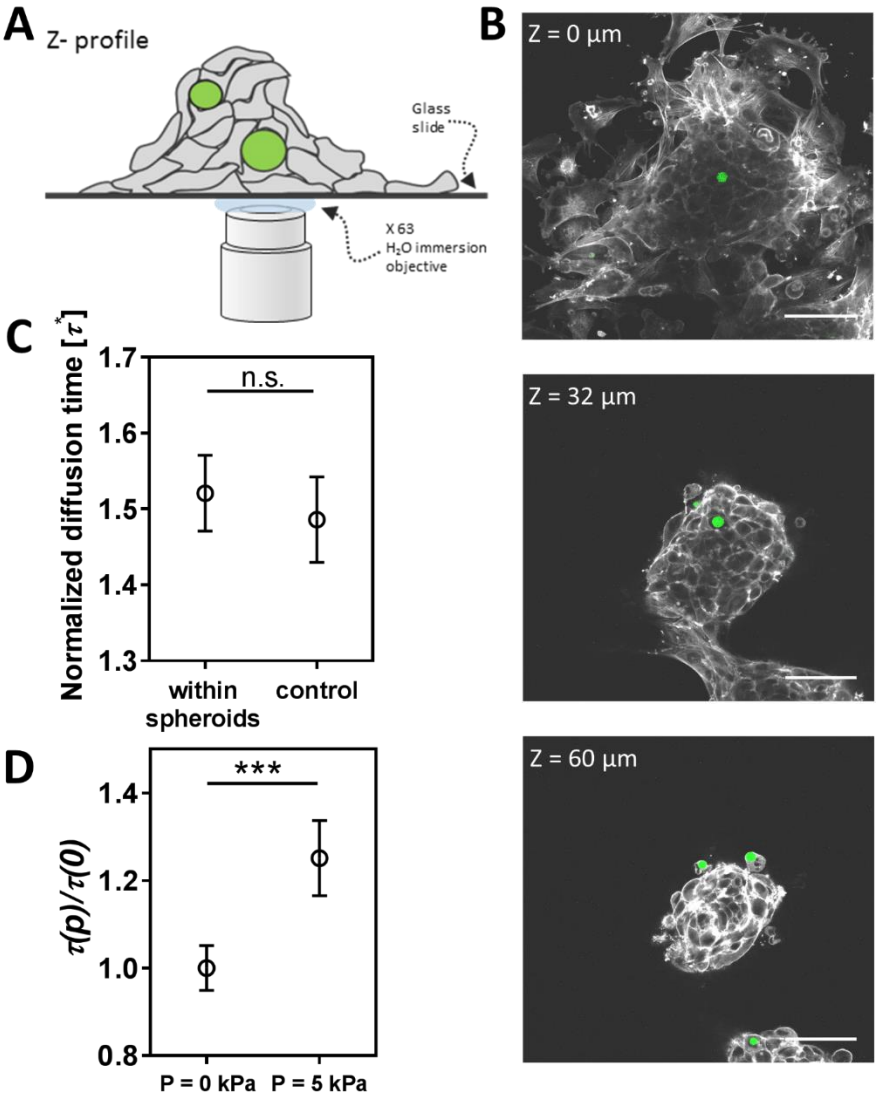

Figure 4 PAA biosensors within the spheroids. A) Schematic representation of the spheroids that spread on surface after 24 hours of culture on a glass substrate coated with poly-L-lysine. B) Series of confocal Z-section images of spheroids (actin in white; beads in green) at the interface with the glass (Z = 0 µm), in the middle (Z = 32 µm), and almost at the top of the spheroid (Z = 60 µm). Scale bar 50 µm. C) Measuring the pre-constraints imposed by the spheroids. Graph presents the normalized characteristic diffusion time of SRB inside beads either incorporated in between cells (within spheroids) or beads outside the spheroids (control). Error bars (S.E.M). (N=8 for the control; N=16 for beads within the spheroids). D) Effect of externally applied isotropic compressive stress. Graph presents the normalized diffusion time (τ(P)/τ(0)) obtained within the beads incorporated within spheroids at P = 0 kPa (τ(0)) and the same beads after an external stress of P=5kPa (τ(P)) was applied. N=5; Error bars (S.E.M). For C) and D) we have used an unpaired t-test and obtained a p=0.1336 (n.s.) for C) and p=0.0001 (***) for D).

Supplementary information

Materials and methods

PAA beads preparation

Polyacrylamide microbeads used in experiments were fabricated using a water-in-oil emulsion approach. Polymerization of 1 mL of a mix of acrylamide and bisacrylamide, at the ratio (x_a, x_b) has been initiated by catalytic activity of TEMED (Sigma, 0.75 uL) with an oxidizing agent APS (Sigma, 10 µL ammonium persulfate, at 10% w/v dissolved in PBS). For imagining purposes FITC-dextran 500 kDa has been introduced to the PAA mixture prior to polymerization. We have additionally added 10µL of acrylic acid (Sigma Aldrich) to promote EDC functionalization. To form an emulsion of acrylamide/bisacrylamide mix in oil, we used HFE 7500 perfluorinated oil (3M) with PFPE-PEG surfactant (kindly provided by Prof. Garstecki). All solutions were degassed for 15 minutes in the vacuum chamber prior use. The mix of polymerizing acrylamide and oil with surfactant has been vigorously shaken in a glass centrifuge tube by vortexing to achieve droplets with diameter ranging from few micrometres up to ~100µm. We kept emulsion under argon atmosphere for 5 minutes and and later during incubation at 60°C for 1h50 . To separate emulsion and transfer beads into PBS, we used 1H, 1H, 2H, 2H-perfluorooctanol (PFO, Sigma Aldrich). Briefly on the top of the emulsion, 500 µL of PBS were gently added with a subsequent addition of 400 µL of PFO. After incubation for 5 minutes, by gentle pipetting the PBS phase, droplets were transferred into the water phase. Fabricated beads were washed thoroughly with PBS to remove excess of non-crosslinked acrylamide monomers. Beads were filtered on 40 µm cell drainer for incorporation within spheroids.

For experiments with spheroids PAA beads were functionalized with fibronectin by EDC protein coupling. First beads were centrifuged in an ultra-low adhesive eppendorfs for 7 minutes at 2000 rpm and washed 3 times with MES buffer (ThermoFisher). Subsequently, beads were incubated with EDC (26 mg/ml) in a MES buffer for 2 hours under gentle agitation. After, beads were washed 3 times with MES buffer and incubated over night with fibronectin (Sigma Aldrich) at the concentration of 40 µg/ml. To remove excess of fibronectin beads were washed 6 times with PBS.

Flat PAA gels preparation

Flat gels of different compositions were prepared by covering a 55 µL drop of acrylamide/bisacrylamide mix residing on a non-treated coverslip (32 mm diam.) with a pre-silanized coverslip (to promote PAA crosslinking, GE Healthcare Life Sciences, Plusone Bind-Silane) (25 mm diam.). PAA mix has been let to polymerize during 30 minutes at room temperature. Non-treated coverslips were gently detached from a PAA gel that rested attached to the silanized coverslip in the presence of water. Gels were immersed in PBS during 3 days. The thickness of the gel was approximately of 60 µm. One night before experiments, the gels were immersed in a SRB solution. This ensured that the SRB concentration in the gel was homogeneous.

In order to determine the volume fraction of PAA, 1 ml samples of gel were prepared in centrifugal tubes. Subsequently gels were removed from the tube and immersed in PBS for 3 days to reach equilibrium. To remove excess of water gels were wiped with a non-absorbing paper and its weight m_{wet} was measured. Afterwards, gels were dried for 3 days in an oven at 70 °C and the new weight m_{dry} was measured. The volume fraction of polymer for the gels at swelling equilibrium has been calculated using $\phi = m_{dry}/m_{wet}$, neglecting the difference in density between polymer and water.

We denote ϕ_{mix} the initial volume fraction of monomer in the mixture before polymerization and ϕ_0 the one at equilibrium after swelling of the gel in PBS. When plotting ϕ_0, the polymer volume fraction after swelling as a function of ϕ_{mix} (see Figure S3), we observe that except for the gel (8, 0.48), all the gels are swelling after the preparation. Indeed the polymer volume fraction is smaller than the volume fraction of the monomer. The (8, 0.48) is obviously releasing water during the polymerisation, since the polymer volume fraction measured after swelling is higher than the monomer fraction in the solution prepared by mixing the compounds. Figure S3 also shows that increasing the acrylamide concentration of the mixture gives a gel which swells more if the cross-linker concentration is kept constant.

Cell culture and spheroid formation

Mouse embryonic fibroblasts (were kindly provided by Dr. Destaing) were maintained in DMEM complete medium enriched with 10% fetal bovine serum (Life Technologies 61965-026) under 95% air and 5% CO2 atmosphere.

Spheroids were prepared using agarose cushion method. Briefly 100 µL of agarose (ultrapure agarose, Invitrogen) was dispensed in wells of 96-well plate and let to polymerize at 4'C for 10 minutes. Subsequently 300 cells were dispensed in each well in 150µL of culture medium. Plates were centrifuged at 900 rpm for 5 minutes to accelerate the process of aggregation. After 48 hours of incubation spheroids were ready to use.

To internalize microbeads within the volume of spheroids, beads were added with the single cells solution at the stage of spheroids preparation at the concentration of approximately 15 beads per well.

For pressure measurements using FCS, spheroids were transferred into 8 well Labtek II plates and let to spread partially for 12 hours. The culture medium is supplemented with Sulforhodamin B (SRB, Radiant Dyes Laser) molecules, at concentration ≈ 20 nM. Before running experiments, the culture medium is replaced by a modified version of DMEM without $NaCO_3$, pyruvate, red phenol and supplemented with 10% FBS and 10mM HEPES (PAA Laboratories GmbHed).

Image acquisition

We used a Leica inverted confocal microscope (DM-IRB; Leica Microsystems, Bannockburn, IL) with 40X oil objective (numerical aperture 1.3) to observe 3D organization of cells within spheroids and around incorporated beads. For bulk modulus measurements we acquired images using an inverted Nikon Eclipse microscope equipped with a 20X dry objective (NA 0.5) and an Andor NEO camera.

FCS measurements

The fluorescence correlation spectroscopy (FCS) measurements were performed on a homemade confocal microscope based on an Olympus IX 71 platform, using a 63X, NA 1.2 water immersion Zeiss objective. Excitation was performed with a diode pumped solid state laser (Cobalt, Solna, Sweden) at 561 nm with a power of a few tens of µW in the sample. Fluorescence was first filtered, using a long pass dichroic mirror at 600 nm (Chroma) and then focused on a 50 µm multimode fibre that acts as a pinhole to be detected with an avalanche photodiode. Optical adjustments were performed in the solution surrounding the beads, the multicellular aggregates or the gel, as the observation was done just above the cover slide. The autocorrelation curves of hydrophilic molecules sulforhodamine B (SRB) at concentrations ≈ 20 nM in PAA gel and beads, were obtained by averaging 2 to 4 acquisitions, lasting 20 s each. They looked like normal diffusion, but slower than in solution, because of hydrodynamic interactions between the molecules and the polymer chains[12], [11]. The averaged autocorrelation functions, weighted with SEM, were fitted for times longer than 2 µs, with a one component FCS model and a fixed triplet time of 2 µs, in order to estimate the number of molecules N and especially the diffusion time τ[13, 14]. In case of beads embedded in multicellular aggregates, it was necessary to correct for photobleaching before autocorrelating, to avoid a bias of the diffusion time[15].

Compression measurements – osmotic stress on beads and spheroids

To determine mechanical properties of PAA microbeads, beads were exposed to a number of osmotic pressures controlled by the concentration of dextran (MW 1.5-2 MDa, Sigma) in the solution. Given the hydrodynamic radius = 27 nm of these dextran molecules and the pore size of the hydrogels (less than 17 nm [16]), the molecules are large enough not to penetrate within the polyacrylamide beads. For each pressure condition, deformation of more than 10 beads was measured. Briefly, focus has been adjusted at the equatorial plane and the diameter has been measured, either manually for non-fluorescent beads, or automatically for FITC-dextran containing beads (IsoData threshold, ImageJ). For simplicity, we define the strain as positive by $(V_0 - V)/V_0$.

Similarly to beads, spheroids were exposed to an isotropic compressive stress by supplementing the medium with a concentration of the same large biocompatible polymer than for beads[7].

Compression measurements – mechanical deformation with a steel bead

To deform gel locally in a controlled manner we used steel beads of mass 55.2 mg and diameter 2.38 mm. Briefly, steel beads were gently deposited on the top of flat PAA gels and the vertical deformation (Δh) has been measured by recording confocal images on a vertical plane with an exposure time of 2 s. The upper and lower surfaces are detected by using a threshold method. Moreover, the characteristic diffusion time has been measured in control region (no deformation) and beneath the bead where the gel was compressed. In this case, the strain was calculated as $(h_0 - h)/h_0$ assuming only a vertical deformation

Supplementary figures

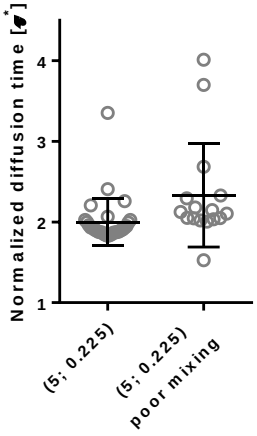

Figure S1 ***Graph illustrates the importance of the PAA beads fabrication protocol. For optimized conditions the characteristic diffusion time varies little, as compared for the batch were mixing was insufficient. N=20, Error bars S.E.M***

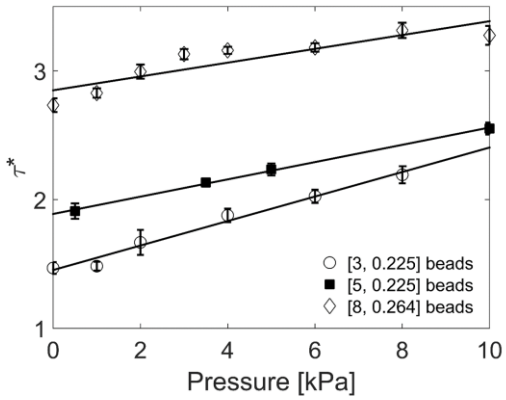

Figure S2 ***Normalized diffusion time calibration curves for different PAA microbeads compositions: (Acrylamide %, Bisacrylamide %) (3, 0.225), (5, 0.225), and (8, 0.264). Error bars are S.D. The linear fits (τ=A Pressure + B) are as follows: gel (3, 0.225): A = 0.095 ± 0.006, B = 1.45 ± 0.03, R^2 = 0.98; gel (5, 0.225): A = 0.067 ± 0.002, B = 1.89 ± 0.01, R^2 = 0.99; gel (8, 0.264): A = 0.05 ± 0.01, B = 2.85 ± 0.06, R^2 = 0.81***

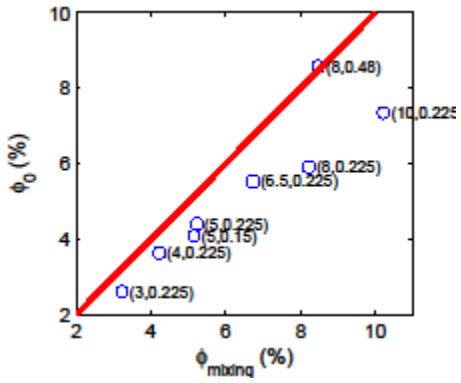

Figure S3: ***plot of the measured polymer mass fraction ϕ_0 after swelling, as a function of the initial mass fraction ϕ_{mix} of the gel before swelling. The acrylamide and bis-acrylamide initial compositions are noted in percent. The red line corresponds to the case where both volume fractions are the same.***