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What Caging Force Cells Feel in 3D Hydrogels: A Rheological Perspective

Giuseppe Ciccone, Oana Dobre, Graham M. Gibson, Jose Manuel Rey, Cristina Gonzalez–Garcia, Massimo Vassalli, Manuel Salmeron–Sanchez, and Manlio Tassieri**

It has been established that the mechanical properties of hydrogels control the fate of (stem) cells. However, despite its importance, a one-to-one correspondence between gels' stiffness and cell behavior is still missing from literature. In this work, the viscoelastic properties of poly(ethylene-glycol) (PEG)-based hydrogels are investigated by means of rheological measurements performed at different length scales. The outcomes of this work reveal that PEG-based hydrogels show significant stiffening when subjected to a compressional deformation, implying that conventional bulk rheology measurements may overestimate the stiffness of hydrogels by up to an order of magnitude. It is hypothesized that this apparent stiffening is caused by an induced "tensional state" of the gel network, due to the application of a compressional normal force during sample loading. Moreover, it is shown that the actual stiffness of the hydrogels is instead accurately determined by means of both passive-video-particle-tracking (PVPT) microrheology and nanoindentation measurements, which are inherently performed at the cell's length scale and in absence of any externally applied force in the case of PVPT. These results underpin a methodology for measuring hydrogels' linear viscoelastic properties that are representative of the mechanical constraints perceived by cells in 3D hydrogel cultures.

G. Ciccone, O. Dobre, J. M. Rey, C. Gonzalez–Garcia, M. Vassalli, M. Salmeron–Sanchez, M. Tassieri
Division of Biomedical Engineering
James Watt School of Engineering
University of Glasgow
Glasgow G12 8LT, UK
E-mail: Manuel.Salmeron-Sanchez@glasgow.ac.uk; Manlio.Tassieri@glasgow.ac.uk

O. Dobre, J. M. Rey, C. Gonzalez–Garcia, M. Vassalli, M. Salmeron–Sanchez
Centre for the Cellular Microenvironment
University of Glasgow
G12 8LT, UK

G. M. Gibson
SUPA
School of Physics and Astronomy
University of Glasgow
Glasgow G12 8QQ, UK

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Over the past two decades, a strong link has been established between the biophysical (i.e., mechanical) properties of cell–culture substrates and cell fate.[1–5] Nonetheless, despite its importance, a one-to-one correspondence between gels' stiffness and cell behavior remains undetermined. Hydrogels have become the most popular materials to study such a relationship because of their inherent simplicity in terms of constituents and preparation, allowing a fine control of their chemical and physical properties, including to mention but a few, stiffness, porosity, and degradability. Hydrogels may be broadly classified into naturally or synthetically derived materials, for example, those based on either proteins and polysaccharides (such as collagen and alginate) or synthetically derived polymers (such as PEG), respectively. Naturally occurring polymers show a high degree of biocompatibility, with some of them being themselves constituents of the extracellular matrix (ECM), whereas synthetic polymers offer the advantage of being rationally designed based on well-defined structural units (i.e., monomers), allowing an accurate control of their biophysical properties. Indeed, their inherent versatility has led to a conceptual shift in their design, from a simple requirement of biocompatibility to the development of bioactive materials capable of interacting dynamically with their environment and orchestrating cellular functionality such as adhesion, differentiation, proliferation, and viability.[3,6–8] A strategy to achieve this has been the incorporation of bioactive molecules such as fibronectin and laminin (LM) within the hydrogel network to mimic the natural cellular microenvironment (where growth factors are bound to the ECM via glycosaminoglycans and other structural proteins) and enhancing the hydrogel effectiveness when presented to the site of an injured tissue.[9]

Despite their successful applications in tissue engineering and regenerative medicine, it remains difficult to draw a line between the synergistic effects of hydrogels' biochemical and biophysical properties on cell behavior, leaving undetermined the relationships between cell fate and the stiffness of their microenvironment. It is known that cells interact mechanically with their environment in a bidirectional way, that is, they are able to exert forces and to perceive them from their surroundings.[10] While the exact molecular pathways governing this interplay are being

determined only recently, the mechanical exchange of forces between the intra- and extracellular environments is governed by transduction pathways involving mechanosensitive receptors,[11] where integrins have been explored as the main transmembrane mechanosensitive receptors.[12,13] When required, integrins transition from passive to active state, reinforcing their bonds to ECM proteins, and ultimately leading to the formation of focal adhesion (FA) complexes, which are large macromolecular assemblies, often of several square micrometers in area, bridging the actin cytoskeleton and integrins via structural and signalling proteins. Therefore, cells are able to "pull" their surrounding environment via forces that are generated within the cells by means of the well-known acto-myosin interactions and transduced to the surrounding by FAs. In turn, cells "sense" the surrounding's elastic response by means of the same biotransducers, which in response activates intracellular processes.[10,14,15]

In this regard, hydrogels have been often used to investigate cell behavior as a function of gels' stiffness,[16–20] with most of the works developed on 2D substrates,[1] and only recently in 3D culture conditions that better resemble the natural environment of the ECM.[3,21] These studies have shown a variety of cell responses as a function of gels' stiffness with variations in morphology,[3,22] spreading,[16,23,24] and fate.[3,25–27] Moreover, it has been shown that stem cell differentiation is strongly governed by the mechanical properties of the surrounding environment, more than any other biochemical factor.[28,29] Nonetheless, there is no consensus on a one-to-one correspondence between absolute values of gels' stiffness and cell behavior, and a clear relationship is still missing in literature. In this study, experimental evidence revealing a possible cause of such lack of information is provided. In particular, the mechanical properties of a series of PEG hydrogels have been investigated by means of i) bulk rheology measurements performed under different normal force conditions, ii) PVPT microrheology measurements inherently performed at zero normal force, and iii) nanoindentation measurements involving sub-μN normal force. This work reveals that PEG-based hydrogels show a significant compressional strain-stiffening behavior, implying that conventional bulk rheology measurements may overestimate hydrogels' stiffness by up to an order of magnitude because of an induced "tensional state" of the gel network, due to the application of a normal force during the sample loading procedure. These findings have been corroborated by a direct comparison with PVPT microrheology and nanoindentation measurements, which both return values of the elastic shear modulus comparable in magnitude to those obtained with bulk rheology measurements performed at relatively low normal forces. Measurements were performed on both degradable and non-degradable PEG-based hydrogels at PEG concentrations ranging from 3.5% to 15% w/v, which translates in a range of mechanical properties that recapitulate those of a broad variety of soft tissues.[3,16,22–27] Degradable hydrogels were obtained by incorporating the protease-cleavable peptide crosslinker GCRDVPMSMRGGDRCG (VPM), whereas non-degradable hydrogels were obtained by incorporating the linear crosslinker PEG–dithiol (HS–PEG–SH) (see Supporting Information and Figure S1 for details). PEG hydrogels have been extensively used in literature because of their hydrophilicity, biocompatibility, and tunable mechanical properties.[30] Moreover, PEG can be easily modified with biofunctional moieties, allowing the incorporation of ECM proteins such as LMs.[31–35] The latter are high-molecular-weight (400 to 900 kDa) heterotrimeric ECM glycoproteins (composed by α, β, and γ subunits, specific to each LM isomer and arranged in a cross-like configuration) present in the basal lamina (or basement membrane) of most tissues. They are known to influence numerous cellular processes such as adhesion, differentiation, migration, and survival via integrin-mediated interactions,[36–38] thus their potential to accelerate the healing process in the presence of tissue defects and our interest in gathering a full picture of the mechanical properties of PEG-based hydrogels for future tissue engineering applications.

The rheological properties of PEG-based hydrogels were investigated by means of strain (γ) sweep tests, with γ ranging from 0.01% to 1% at an angular frequency (ω) of 10 rads^{-1} (see Supporting Information). A stress controlled rheometer was equipped with a parallel plate of 15 mm in diameter. Measurements of the gels' shear elastic modulus (G') were performed by gradually varying the normal force applied to the unconfined samples, starting from a minimum force value of ≈0.01 N and with a minimal delay (i.e., of the order of a few seconds) between sequential compressions. From **Figure 1**, it is clear that the mechanical properties of PEG-based hydrogels are strongly affected by the presence of a normal force, which induces a variation of G' by up to an order of magnitude. Notice that, this issue would not occur in rheological studies of viscoelastic fluids, for which a sufficiently long time is given to the sample to fully relax after the loading procedure; this is an unachievable requirement for solid-like materials (e.g., rigid gels), which have (in theory) an infinite time of relaxation. The increase of G' as a function of the normal force is shown by all the PEG systems investigated in this work, both degradable and non–degradable systems and regardless of the functionalization with LM, as reported in Figure 1. The hypothesis is that such increase of G' is due to a 3D deformation of the polymeric network of the gels due to the application of a compressional load, resulting in an induced tensional state of the network and ultimately in a compressional strain–stiffening phenomenon.[39,40] Similarly, shear strain–stiffening is a nonlinear mechanical behavior of materials showing an increase in G' as they are subjected to increasingly large shear strain.[41–44] This is common to many biological materials, preventing damage to large deformations that would otherwise undermine tissue structure and functionality,[39,41,42] as supported by the experimental studies performed on both natural (e.g., soft fibrin and collagen gels)[41,42] and synthetic hydrogels.[43,44] Interestingly, when a uniaxial compressional strain is coupled to a shear strain, soft collagen and fibrin gels have shown compressional softening, while still holding their shear strain–stiffening behavior,[45] whereas native tissues such as adipose and brain tissues have shown both compressional and shear strain–stiffening.[39] This behavior has been experimentally and computationally demonstrated to be reversible by introducing stiff particles within the gels, so that the mechanical properties of native tissue could have been closely mimicked.[39,40] To the authors' best knowledge, the compressional strain–stiffening behavior of synthetic hydrogels, such as those investigated in this work (also widely used within the tissue engineering community), has never been explored. From Figure 1, it is interesting to notice that such phenomenon becomes more pronounced at higher concentrations of PEG. This behavior is inline with the increase of the number of

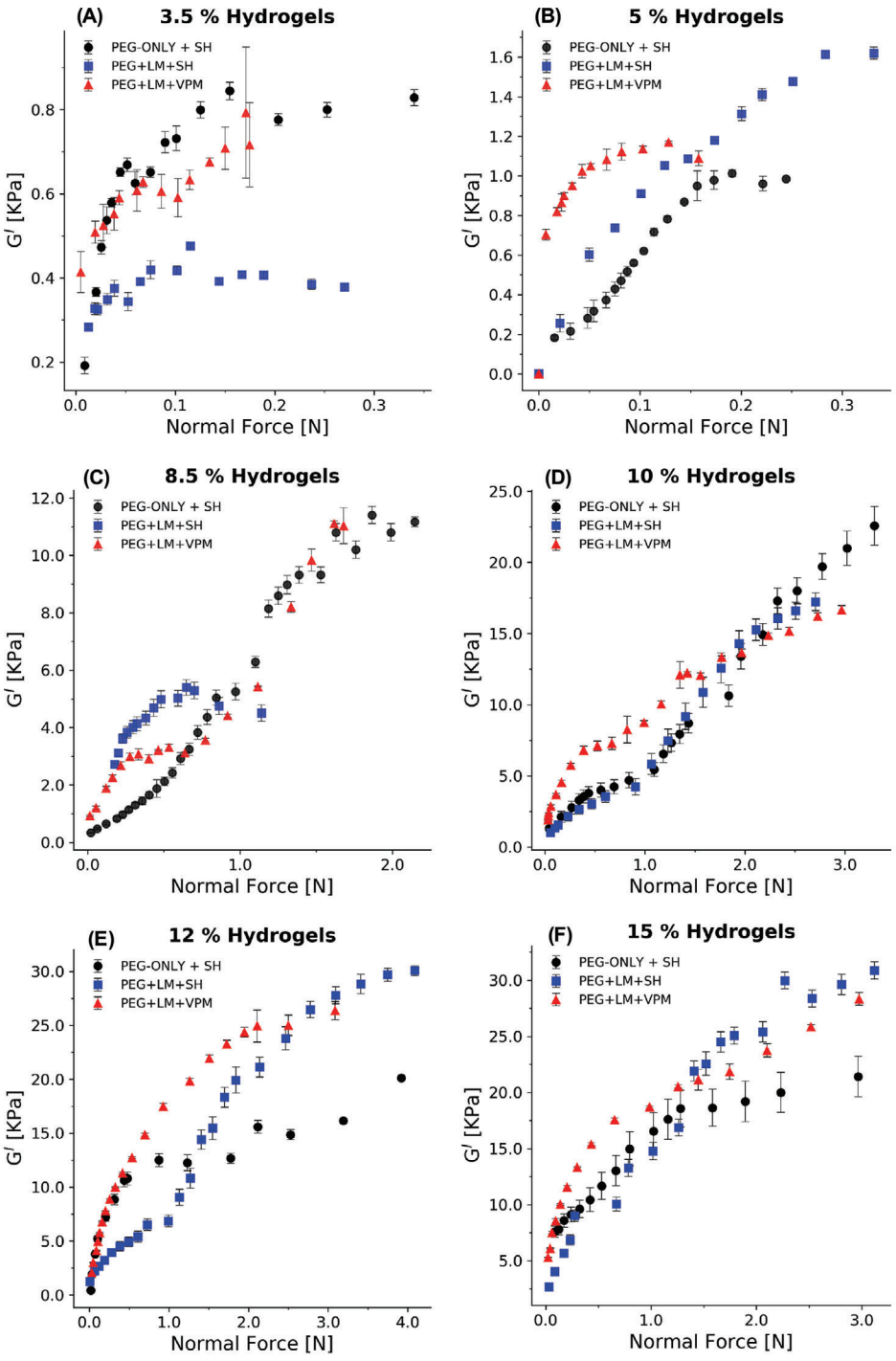

Figure 1. The shear elastic modulus (G') versus the normal force measured via bulk rheology measurements for i) PEG–ONLY non–degradable hydrogels (PEG–ONLY+SH, black circles), ii) PEG–LM non–degradable hydrogels (PEG+LM+SH, blue squares), and iii) PEG–LM degradable hydrogels (PEG+LM+VPM, red triangles) for A) 3.5% w/v, B) 5% w/v, C) 8.5% w/v, D) 10% w/v, E) 12% w/v, and F) 15% w/v PEG. Each data point is the mean of at least three consecutive values of G' at low strains (≈0.01%) measured under a constant normal force (see Supporting Information). Error bars report ± 3SD, $n = 1$ per hydrogel.

cross-links within the network, which would promote stress propagation throughout the gel[46] but also with the fact that softer gels show a strain–stiffening behavior only for stress values higher than the material's rupture stress.[44] To reiterate, we hypothesize that the presence of a normal load causes a 3D deformation of the network, which results into an induced tensional state and ultimately in a compressional strain–stiffening phenomenon. In order to support such hypothesis and to rule out the contribution of other possible processes that may induce a similar behavior of the gels, such as the loss of water during the

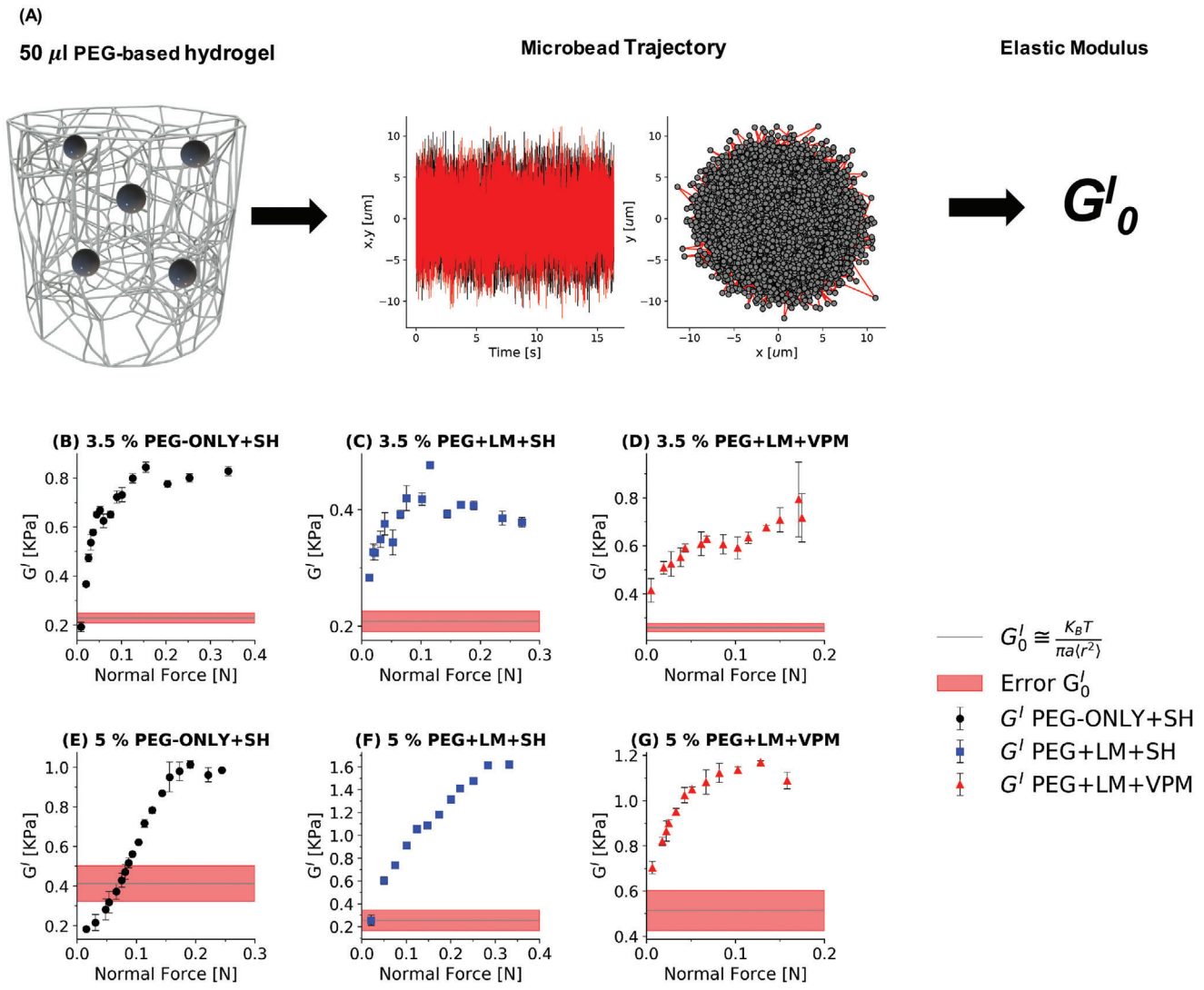

Figure 2. Comparison between bulk and microrheology measurements. A) A typical PVPT microrheology experiment. On the left, a schematic of a PEG–based hydrogel laden with microspheres is shown. The stochastic motion of the beads is recorded at high spatial and temporal resolution (center) and converted into its MSD. The statistical mechanics analysis of the bead trajectory has the potential of revealing the rheological properties of the surrounding media (see Supporting Information). B–G) Comparison between the shear elastic modulus, G', probed via bulk rheology measurements performed at different applied normal forces, with the shear elastic modulus, G'_0, obtained via PVPT microrheology for the hydrogels listed in Table 1. Bulk rheology: Each data point is the mean of at least three consecutive values of G' at low strains ($\approx$0.01%) measured under a constant normal force (see Supporting Information). Error bars report ± 3SD, $n = 1$ per hydrogel. Microrheology: The mean (gray line) and standard error (red band) have been evaluated on at least four independent measurements, $n = 1$ per hydrogel.

compression of hydrogels, the same tests were performed on a dry, highly porous polymeric material (i.e., a synthetic sponge) having a diameter of 17.2 mm, thickness 3 mm (similar to the PEG-based samples) and by using two different parallel-plates having diameters of 15 and 25 mm, to discard boundary effects, too. These results are reported in Figure S2, Supporting Information, from which it can be seen that i) the synthetic dry network shows the same behavior as the highly hydrated gels, and ii) a similar behavior is obtained when the sample is contained within the parallel plates, without the risk of excreting them nor of edging effects. Moreover, in order to validate that compressional strain–stiffening is a general property of 3D synthetic polymer networks (regardless of their chemical formulation), similar tests were performed on hydrogels that have been widely used in literature, including: a polyacrylamide (PA) hydrogel,[47,48] a PEG-malemide (MAL) hydrogel,[49] and a polydimethylsiloxane (PDMS) rubber[50] (Figure S3, Supporting Information). Notably, all the tested gels show similar behavior as the PEG-based hydrogels reported in this work, corroborating that compressional strain–stiffening is a common property of synthetic hydrogels. Further, the same methodology has been employed to shed some light on a dispute over the effects of the addition of LM to PEG hydrogels.[32–35] In particular, from Figure 1, at low normal force values, LM has a significant hardening effect on softer PEG hydrogels (i.e., at PEG concentrations lower than 8.5% w/v), in-line with LM introducing more cross-linking points and leading

Table 1. The shear elastic modulus measured via PVPT microrheology (G'_0) and the minimum shear elastic modulus measured via bulk rheology (G'_{min}).

Figure 2	Hydrogel	$G'_0 \pm$ Error [Pa]	$G'_{min} \pm$ Error [Pa]	$G'_{Nano} \pm$ Error [Pa]
B	3.5% PEG–ONLY+SH	228 ± 21	191.60 ± 19.62	230 ± 10
C	3.5% PEG+LM+SH	207.91 ± 17.68	283.3 ± 4.6	–
D	3.5% PEG+LM+VPM	258.96 ± 20.78	413.9 ± 49	–
E	5% PEG–ONLY+SH	411.77 ± 89.47	183.3 ± 9.2	220 ± 20
F	5% PEG+LM+SH	252.88 ± 42.40	256 ± 45.3	–
G	5% PEG+LM+VPM	513.32 ± 92.28	703.2 ± 27.5	–

[a] Nanoindentation measurements performed on PEG hydrogels at two concentrations are reported (G'_{Nano}). G'_{min} and G'_0 (and associated errors) have been obtained as described in Figure 1 and 2. G'_{Nano} is reported as mean ± 1SD from a set (> 20, see Supporting Information) of single indentation curves; $n = 1$ per hydrogel per technique.

to a stiffer network (see Figure S1, Supporting Information),[46] whereas this effect becomes less apparent for hydrogels with a higher content of PEG. Interestingly, this is not the case for PEG–LM hydrogels where the SH component has been replaced with VPM, for which a significant stiffening of the hydrogels is shown when compared to those made of PEG-ONLY, at all the explored concentrations.

It is now possible to draw some preliminary conclusions based on the experimental evidences reported in Figure 1, but also on those reported by a multitude of works aimed at finding a yet unknown one–to–one correspondence between gels' stiffness and cell behavior, and from which a simple question arises: what stiffness do cells actually feel in 3D hydrogels? A possible answer may come from PVPT measurements, which it is reiterated are performed at the same length scales of cells and they are thermally driven, that is, they do not require the use of any externally applied force to induce a sample deformation. In brief, the underlying principles of PVPT microrheology are based on the statistical mechanics analysis of the thermal fluctuations of microspheres embedded into the complex material under investigation, as shown in **Figure 2**A (and described in Supporting Information). At thermal equilibrium, PVPT microrheology measurements have the potential of revealing the frequency-dependent linear mechanical properties of the surrounding media, which in the case of solid-like materials (like gels) can be narrowed down to a single component defined by the (almost) frequency-independent shear elastic modulus G'.[51–53] In Figure 2B–G are compared the results obtained from PVPT microrheology and bulk rheology measurements, with the latter performed under different loadings of normal force. Notice that, only relatively soft hydrogels were tested with PVPT, namely those at PEG concentrations of 3.5% and 5% w/v. This is because stiffer hydrogels would dampen the thermal fluctuations of the probe particle below the spatial resolution of the detector. It is hypothesized that cells cultured in 3D hydrogels must feel a stiffness that is closer to that measured by PVPT microrheology (Figure 2). This means a methodology involving normal forces close to zero has to be used when measuring the elasticity of synthetic hydrogels using bulk rheology measurements (**Table 1**). The relatively small discrepancy between the two methods can be ascribed to the resolution of the transducer used for the detection of the normal force in bulk rheology measurements, namely 0.005 N in this study. Nonetheless, nanoindentation measurements performed on PEG hydrogels at two concentrations (i.e., 3.5% and 5% w/v PEG-ONLY + SH) returned values of the shear elastic modulus of the same order of magnitude as those of bulk rheology measurements performed at low normal forces (Table 1).

To conclude, it is possible to argue that the multi-scale rheological characterization of PEG hydrogels presented in this work provides a possible justification for the yet undetermined one-to-one correlation between the stiffness of commonly used synthetic cell culture gels and cell fate. Indeed, the experimental evidences provided in this work reveal that bulk rheology measurements could overestimate the mechanical properties of synthetic hydrogels by up to an order of magnitude, if attention is not paid to the level of applied normal force during the sample loading procedure,[54] making it difficult to quantitatively correlate the stiffness of the substrate to cell behavior. It is argued that this is due to an induced tensional-state within the gel network due to the application of a compressional normal force during sample loading, a phenomenon itself of particular importance when considering implantation of hydrogels in vivo, as most tissues exist in a pre-stressed state[55] and show compressive strain–stiffening behavior.[39] Finally, it is envisaged that a synergistic implementation of nanoindentation, microrheology, and bulk rheology measurements performed at low normal forces may uncover a new strategy for the mechanical characterization of biomaterials for biomimetic culture platforms aimed at reproducing in vitro tissue-realistic cell behavior.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

hydrogels, microrheology, poly(ethylene-glycol), rheology, tissue engineering

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