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Supplementary Information for Compressional stress stiffening & softening of soft hydrogels – how to avoid artefacts in their rheological characterisation

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Hydrogels have been successfully employed as analogues of the extracellular matrix to study biological processes such as cells' migration, growth, adhesion and differentiation. These are governed by many factors, including the mechanical properties of hydrogels; yet, a one-to-one correlation between the viscoelastic properties of gels and cell fate is still missing from literature. In this work we provide experimental evidence supporting a possible explanation for the persistence of this knowledge gap. In particular, we have employed common tissues' surrogates such as polyacrylamide and agarose gels to elucidate a potential pitfall occurring when performing rheological characterisations of soft-materials. The issue is related to (i) the normal force applied to the samples *prior* to performing the rheological measurements, which may easily drive the outcomes of the investigation outside the materials' linear viscoelastic regime, especially when tests are performed with (ii) geometrical tools having unbefitting dimensions (i.e., too small). We corroborate that biomimetic hydrogels can show either compressional stress softening or stiffening, and we provide a simple solution to quench these undesired phenomena, which would likely lead to potentially misleading conclusions if they were not mitigated by a good practice in performing rheological measurements, as elucidated in this work.

Rheological measurements were performed by means of a stress controlled rheometer (Anton Paar Physica MCR 302 Instruments) equipped with three sets of interchangeable parallel plates (i.e., PP08-SN84133, PP15-SN58414, PP25-SN36246) having diameters of 8mm, 15mm and 25mm, which are referred to in Figure 1a as PP08, PP15 and PP25, respectively. Notably, these geometries allowed us to scale by up to an order of magnitude the applied normal force perpendicular to the shear deformation, when converting it into compressional stress. This is because $\sigma_{cPP8}/\sigma_{cPP25} = (D_{PP25}/D_{PP8})^2 \cong 10$, as schematically shown in Figure 1b. The gels' viscoelastic properties were measured at room temperature (22^oC) by performing strain (γ) sweep tests (Figure 1c), with amplitudes ranging from 0.01% to 1% at a constant angular frequency (ω) of 10rad/s. The storage and the loss moduli were measured by gradually increasing the normal force applied to the unconfined samples, starting from a minimum force value of circa 0.01 N and with a minimal delay of the order of a few minutes to ensure the achievement of a stationary normal force between sequential compressions, as corroborated by the three representative measurements reported in Figure S1.

Consecutive squeezing of the samples was achieved by gradually reducing the gap between the parallel plates, which translated into a range of explored compressional axial strain varying from a minimum value of 0.1% to a maximum one of 80%, as shown in Figures S2. Notably, our results are in very good agreement with those reported by Xie *et al.*[1] in Figure 6a of their manuscript for both agarose and polyacrylamide gels and for comparable compressional axial strains (i.e., axial strain $\leq 30\%$).

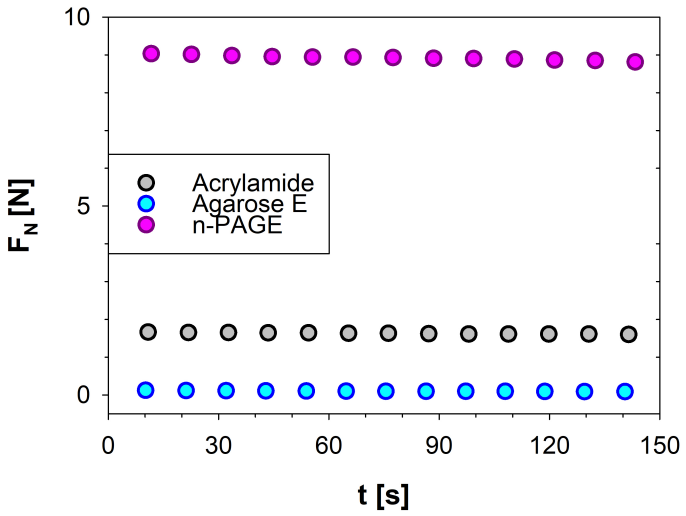

Fig. S1 The normal force F_N versus measurement time, for three representative measurements of polyacrylamide (solution-based and powder-based) and agarose gels, supporting the achievement of a stationary normal force between sequential compressions and therefore during the measurements.

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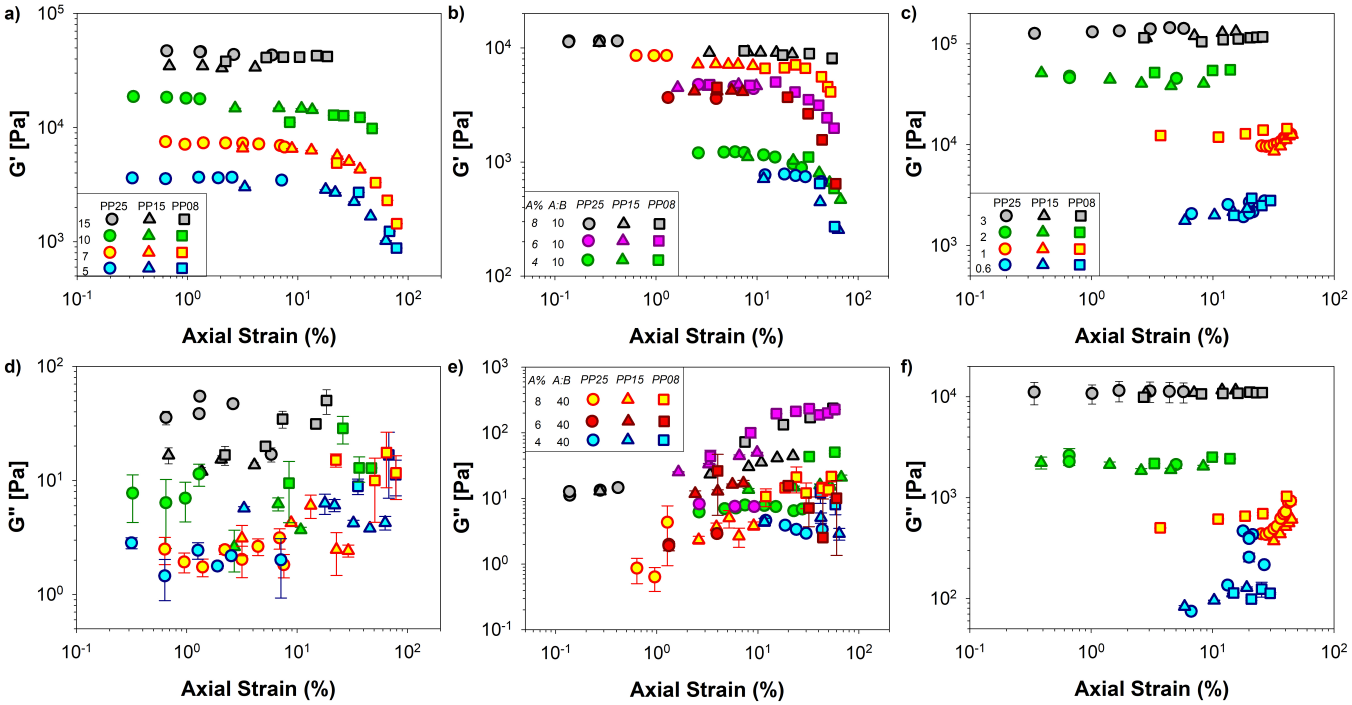

Fig. S2 The shear storage modulus ($G'(\omega)$, top row) and the shear loss modulus ($G''(\omega)$, bottom row) versus the absolute value of the axial strain, for polyacrylamide (solution-based in a-d and powder-based in b-e) and agarose (c-f) gels. The absolute value of the axial strain is defined as: $100 \times |(h - h_0)|/h_0$, where h_0 is the initial gap size at the first contact point detected by the instrument (i.e., when the normal force is $\simeq 0.01$N). Measurements were repeated using parallel plate tools with different diameter, i.e.: 8 mm (square symbols), 15 mm (triangle symbols), 25 mm (circle symbols).

Notes and references

1 Q. Xie, Y. Zhuang, G. Ye, T. Wang, Y. Cao and L. Jiang, *Nature Communications*, 2021, **12**, 4277.