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# 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.

Over the past two decades, hydrogels have attracted a great deal of interest in the field of biomaterials, especially for their applications as scaffolds in tissue-engineering,<sup>1–3</sup> regenerative medicine and surgical training.<sup>2</sup> Their success relies on their easily designable mechanical and physical properties, such as flexibility, transparency and permeability<sup>4</sup>; notwithstanding

their high water content (of up to ~99.9% wt). These properties are of great relevance to a variety of biological and biomedical applications, and they are governed by the structure and the dynamics of hydrogels' three-dimensional (bio-)polymer network. Indeed, owing to their capability of being easily mouldable in three-dimensional structural matrices, with a high degree of biocompatibility, bioinductivity and biodegradability,<sup>5,6</sup> hydrogels have been extensively and successfully used for a broad range of *ex vivo* and *in vitro* applications, such as drug delivery,<sup>7–10</sup> wound healing<sup>11,12</sup> or as artificial tissue.<sup>13–15</sup> Of particular interest to this work are those biomedical studies driven by the aim to gain a better understanding of the effect of the extracellular matrix (ECM) stiffness on cells' behaviour and fate, for which scientists have developed a variety of natural and synthetic hydrogels to mimic both the physiological and the mechanical properties of ECM. In particular, in order to evaluate the effectiveness and the reliability of these biological analogues, an accurate characterization of their mechanical properties is necessary to enable the construction of a one-to-one correlation between gels' viscoelastic properties and cells response in terms of migration,<sup>16–18</sup> growth,<sup>19–21</sup> adhesion<sup>22</sup> and differentiation.<sup>23–25</sup> Hence, the aim of this communication is to inform the scientific community of a potential pitfall when performing a rheological characterization of soft-tissues and gels, as elucidated hereafter.

The viscoelastic properties of hydrogels designed for mimicking biological specimens<sup>26–29</sup> are fully described by their frequency-dependent shear complex modulus  $G^*(\omega) = G'(\omega) + iG''(\omega)$ , which is a complex number whose real ( $G'(\omega)$ , also known as storage modulus) and imaginary ( $G''(\omega)$ , also known as loss modulus) parts provide valuable information on the elastic and viscous nature of the sample, respectively. Conventionally, these are determined *via* oscillatory measurements performed by means of rotational rheometers. These are often equipped with parallel-plates geometries that require millilitres of sample volume to maximise the contact area between the force (*i.e.*, torque) transducer and the sample. However, when testing biological samples, which are commonly exiguous and

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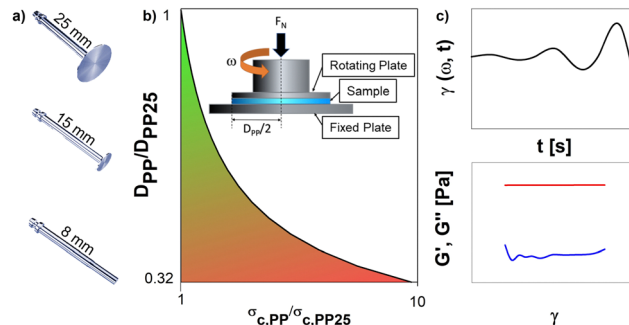
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expensive, rheology measurements are often performed by using relatively small tools (*i.e.*, with geometries having a relatively small contact area) to minimise the sample volume. Unfortunately, this is a common practice that may trigger two potential pitfalls that could compromise the validity of the outcomes of the studies: *i.e.*, (i) a significant reduction in sensitivity of the torque measurements and (ii) the potential increase of compressional stress perpendicular to the shear deformation ( $\sigma_c$ ) applied during the sample loading procedure.<sup>30,31</sup> This latter point is actually the main concern addressed in this communication, for which a simple solution supported by experimental evidence is presented.

As already highlighted in literature,<sup>30–32</sup> compressional stress stiffening and softening of soft materials during rheological measurements may risk to further undermine the yet unmet aim of the scientific community to establish a one-to-one correlation between gels' stiffness and cell fate.<sup>23,33–37</sup> As we shall demonstrate hereafter, a simple and effective solution to minimise the undesired effects caused by a compressional stress perpendicular to the shear deformation during the materials loading and measurements is achieved by employing rheological tools such as parallel-plates with a relatively large contact area. This is because the applied compressional stress is inversely proportional to the square of the tool diameter ( $D$ ):  $\sigma_c = 4F_N/\pi D^2$ , when the applied normal force ( $F_N$ , perpendicular to the shear deformation) is kept constant. Therefore, by performing a preliminary study for the selection of the most appropriate set of parallel-plates for a given soft-material, it is then possible to remove any uncertainty related to the compressional stress.

In order to support our thesis, we have measured the viscoelastic properties of three well-known and commonly used hydrogels at different concentrations and tested each sample with three sets of parallel-plate geometries, within a similar range of applied normal forces. In particular, here we have investigated the rheological properties of common tissues' surrogates, such as polyacrylamide (PAM)<sup>23,38</sup> and agarose<sup>39–41</sup> at concentrations that would produce gels with mechanical properties similar to those of a wide range of biological tissues.<sup>42,43</sup> Specifically, measurements were performed on polyacrylamide gels obtained by Acrylamide/Bis Solution (40% Acrylamide/Bis Solution, 29:1, Bio-Rad, #1610147) or by Acrylamide/Bis in powder at two different crosslinker densities, 10/1 and 40/1, (Acrylamide, Bio-Rad, #1610100 and Bis Crosslinker, Bio-Rad, #1610200) with acrylamide concentrations ranging from 5% to 15% v/v and from 4% to 8% wt, respectively. For both the above cases, 200  $\mu$ l of ammonium persulfate (APS) at 10% w/v were added to a final water-based solution of 20 ml. In order to initiate the polymerization reaction, 20  $\mu$ l of  $N,N,N',N'$ -tetramethylethylenediamine accelerator (TEMED) were used. Measurements were also performed on agarose gels (Agarose E, Condalab, #8100) at concentrations ranging from 0.6% wt to 3% wt. Hydrogels solutions of  $\sim 10$  ml in volume were casted between two glass plates with a spacer of 1.5 mm of thickness (Bio-Rad, Mini-PROTEAN Spacer Plates with 1.5 mm Integrated Spacers, #1653312). After polymerisation, which took approximately 30 minutes at room temperature, glasses were removed, and the gels were pinched



**Fig. 1** A schematic representation of the linear rheology measurements. (a) The parallel plates tools of the rheometer having different diameters, *i.e.*: 8, 15 and 25 mm, referred in the manuscript as PP08, PP15 and PP25, respectively. (b) Variation of the applied compressional stresses as function of the plates diameter, both normalised to the relative value achieved with a PP25 and per unit normal force. The inset is a schematic representation of the parallel plates geometry employed for the rheological characterisation of gels. (c) Schematic representations of an amplitude sweep test (top) and of the shear viscoelastic moduli as a function of shear strain ( $\gamma$ ) (bottom). The latter is drawn in double log scale.

(by means of a circular cutter) to match the diameter of the tools and give them a cylindrical (pill) shape with a thickness of  $1.5 \text{ mm} \pm 0.1 \text{ mm}$ .

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 8 mm, 15 mm and 25 mm, which are referred to in Fig. 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  $\sigma_{c,PP8}/\sigma_{c,PP25} = (D_{PP25}/D_{PP8})^2 \cong 10$ , as schematically shown in Fig. 1b. The gels' viscoelastic properties were measured at room temperature ( $22^\circ \text{C}$ ) by performing strain ( $\gamma$ ) sweep tests (Fig. 1c), with amplitudes ranging from 0.01% to 1% at a constant angular frequency ( $\omega$ ) of  $10 \text{ rad s}^{-1}$ . 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 constant normal force between sequential compressions. These latter compressions 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 Fig. S1 and S2 of ESI†. Notably, our results are in very good agreement with those reported by Xie *et al.*<sup>29</sup> in Fig. 6a of their manuscript for both agarose and polyacrylamide gels and for comparable compressional axial strains (*i.e.*, axial strain  $\leq 30\%$ ).

In Fig. 2a and d, the viscoelastic moduli of both PAM systems mentioned above are reported as function of  $\sigma_c$ . For each sample, multiple measurements were performed within a similar range of applied normal forces, but with parallel plates having different dimensions, thus exploring different  $\sigma_c$ . From



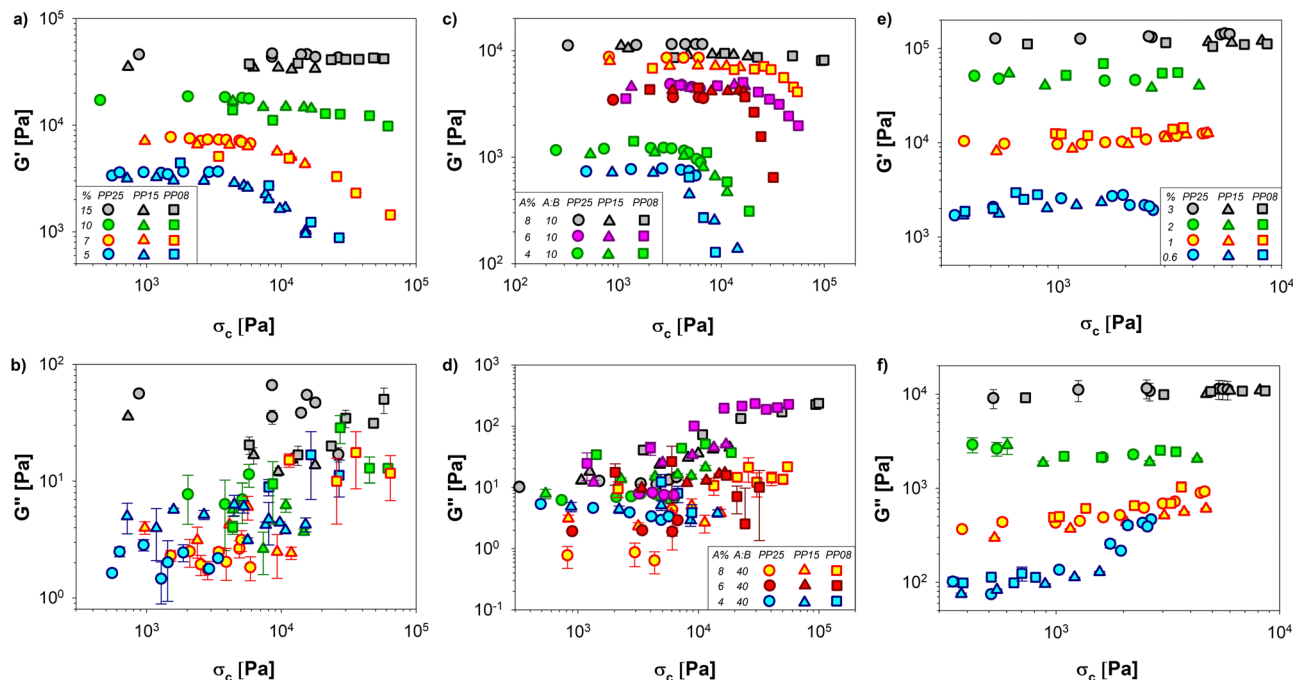


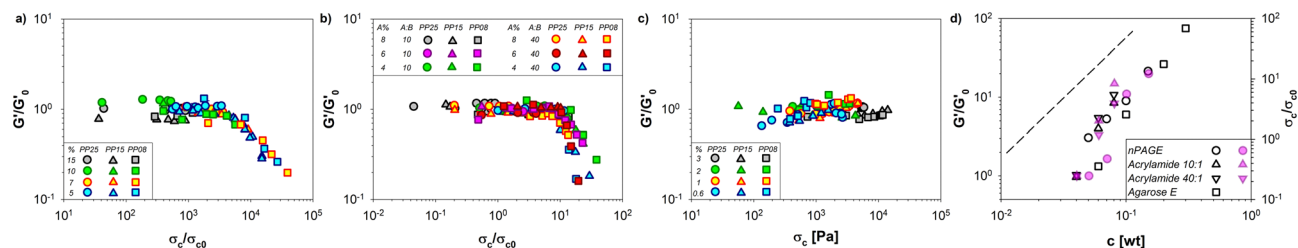
Fig. 2 The shear storage modulus ( $G'(\omega)$ , top row) and the shear loss modulus ( $G''(\omega)$ , bottom row) versus the compressional stress ( $\sigma_c$ ), for polyacrylamide (solution-based in a and b and powder-based in c and d) and agarose (e and f) gels.  $\sigma_c$  is defined as the ratio between normal force and tools area in contact with the sample. Measurements were repeated using parallel plate tools with different diameter, *i.e.*: 8 mm (square symbols), 15 mm (triangle symbols), 25 mm (circle symbols).

the data, it is apparent that both of the moduli are strongly affected by  $\sigma_c$ , but in particular the elastic modulus shows a compressional softening at relatively high values of  $\sigma_c$ ; which are easily achieved when measurements are performed with a PP08, but they are never reached when a PP25 is employed. From Fig. 2a and c it can be seen that the adoption of a PP08 may cause a reduction of  $G'(\omega)$  of almost an order of magnitude in the case of weaker gels when compared to the outcomes obtained with a PP25. In particular, for the hydrogel with the lowest concentration of solution-based PAM (which coincidentally have a degree of elasticity similar to those seen in living systems, *i.e.* cells),  $G'(\omega)$  decreases by a factor of  $\sim 4$  when comparing the outcomes of the PP25 and the PP08, respectively. Whereas, in the case of the powder-based PAM hydrogel,  $G'(\omega)$  decreases by a factor of  $\sim 2.3$  for PAM hydrogel at a crosslinker density of 10% and by a factor of  $\sim 5.2$  for the crosslinker density of 2.5%. Interestingly, as shown in Fig. 2b and d, the loss modulus does not show a similar behaviour to the storage one and a correlation with the applied compressional stress is not apparent. Different is the case of agarose hydrogels, which show a slight compressional stress stiffening behaviour for both of the viscoelastic moduli, especially at low concentrations. Indeed, as shown in Fig. 2e, for the two highest concentrations of agarose,  $G'(\omega)$  is almost constant within the range of explored  $\sigma_c$ ; whereas, it shows a slight increase for the weaker agarose gel, possibly because of an induced tensional state of the polymer network.<sup>44,45</sup> In particular, for the hydrogel with the lowest concentration of agarose (*i.e.*, 0.6% wt),  $G'(\omega)$  increases by a factor of  $\sim 1.6$  and  $\sigma_c$  increases by *circa* one order

of magnitude when moving from PP25 to PP08. Interestingly, the effects of the compressional stress are more apparent for  $G''(\omega)$ , which increases by a factor of *circa* 5 for the same increment of  $\sigma_c$  (Fig. 2f).

The experimental evidence presented so far clearly indicates that the common practice of performing rheological investigations of soft materials at a constant applied normal force, usually adopted as reference for comparing the response of different materials, is not a condition sufficient to guaranty consistency nor reproducibility of the outcomes. Instead, we can thus assert that rheological characterisations of soft materials should better be compared at constant compressional stress. Indeed, as shown in Fig. 2, which has been drawn by means of measurements performed within the same range of applied normal forces, the adoption of a relatively small parallel plates (*i.e.*, here PP08) may cause the measurements to overrun into the materials' non-linearity regime.

In order to further corroborate our findings, we compared them with the existing theoretical scaling laws describing the stiffness of the polymer network as function of the polymer concentration. In particular, in Fig. 3a we report a master curve of the storage modulus *versus* the compressional stress, which has been obtained by shifting the original data shown in Fig. 2a both vertically and horizontally to close match those of the weakest sample taken as a reference ( $\sigma_{c0}$ ,  $G'_0$ ). A similar procedure has been implemented for PAM powder-based hydrogel in Fig. 3b and for agarose hydrogel in Fig. 3c. Interestingly, as shown in Fig. 3c, a horizontal shift (*i.e.*,  $\sigma$ -shift) was not necessary for the agarose hydrogels. Notably, by plotting both the



**Fig. 3** Master curves of hydrogels elastic properties (a and b acrylamide, c agarose): The shear storage modulus  $G'(\omega)$  vs. the compressional stress  $\sigma_c$ , both scaled by the relative values of the weakest sample taken as a reference (*i.e.*,  $G'_0$  and  $\sigma_{c0}$ ). Measurements were repeated using tools with different diameter (square symbols – 8 mm, triangle symbols – 15 mm, circle symbols – 25 mm). (d)  $G'/G'_0$  (open symbols) and  $\sigma_c/\sigma_{c0}$  (filled symbols) are reported as function of concentration for the systems investigated here. The dashed line is guide for the power law:  $|G'| \propto c^2$ .

vertical  $G'/G'_0$  and the horizontal  $\sigma_c/\sigma_{c0}$  shifting factors as function of concentration ( $c$ ) for all the hydrogels investigated in this work (see Fig. 3d), the data follow the well-established concentration scaling-law for the storage modulus  $|G'| \propto c^2$  of crosslinked polymer networks reported by MacKintosh *et al.*<sup>46</sup> Moreover, it is worth mentioning that the experimental procedure presented in this work provides for the first time in literature a means of measuring the compressional yield stress ( $\sigma_{(cy)}$ ) of hydrogels and its concentration scaling law: *i.e.*,  $\sigma_{(cy)} \propto c^2$ .

In conclusion, we can assert that in order to build a one-to-one correlation between gels' viscoelastic properties and cells' behaviour (underpinning biological processes such as migration,<sup>16–18</sup> proliferation,<sup>19–21</sup> adhesion<sup>22</sup> and differentiation<sup>23–25</sup>) attention must be paid to (i) the relative value of the compressional stress applied to the samples prior to performing the measurements and therefore to (ii) the dimension of the geometrical tools (*i.e.* to their contact area with the sample) used for measuring the linear viscoelastic properties of biomimetic hydrogels. Notably, as we shall demonstrate in a follow-up publication, the same principles are transferable to indentation measurements performed with atomic force microscopy instruments, for which the contact area is not constant (*i.e.*, it is 'zero' at the contact 'point' of the cantilever tip with the sample), and a significant variation of the applied compressional stress (starting from an 'infinite' value at the contact 'point') must be taken into account.<sup>47,48</sup> These concepts are of crucial importance for a large scientific community, and our results, together with those recently reported in literature,<sup>30,31</sup> clearly indicate that biomimetic hydrogels can show either softening or stiffening when subjected to a relatively high compressional stress perpendicular to the shear deformation, which would likely lead to potentially misleading conclusions if they were not mitigated by a good practice of rheological measurements, as elucidated in this work.

## Conflicts of interest

There are no conflicts to declare.

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