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Multi-modal characterization of polymeric gels to determine the influence of testing method on observed elastic modulus

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Abstract

Demand for materials that mechanically replicate native tissue has driven development and characterization of various new biomaterials. However, a consequence of materials and characterization technique diversity is a lack of consensus within the field, with no clear way to compare values measured *via* different modalities. This likely contributes to the difficulty in replicating findings across the research community; recent evidence suggests that different modalities do not yield the same mechanical measurements within a material, and direct comparisons cannot be made across different testing platforms. Herein, we examine whether “material properties” are characterization modality-specific by analyzing the elastic moduli determined by five typical biomaterial mechanical characterization techniques: unconfined-compression, tensiometry, rheometry, and micro-indentation at the macroscopic level, and microscopically using nanoindentation. These analyses were performed in two different polymeric gels frequently used for biological applications, polydimethylsiloxane (PDMS) and agarose. Each was fabricated to span a range of moduli, from physiologic to supraphysiologic values. All five techniques identified the same overall trend within each material group, supporting their ability to appreciate relative moduli differences. However, significant differences were found across modalities, illustrating a difference in absolute moduli values, and thereby precluding direct comparison of measurements from different characterization modalities. These observed differences may depend on material compliance, viscoelasticity, and microstructure. While determining the underlying mechanism(s) of these differences was beyond the scope of this work, these results demonstrate how each modality affects the measured moduli of the same material, and the sensitivity of each modality to changes in sample material composition.

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Declarations of interest

None.

1. Introduction

Biomaterial mechanical properties have become increasingly important features to prescribe/control for applications such as drug delivery (Gombotz and Wee, 1998; Hoare and Kohane, 2008; Uhrich et al., 1999), prosthetics, and engineered tissue replacements (Bahraminasab and Jahan, 2011; Cheal et al., 1992; Joshi et al., 2000). Their influence on cell behavior has recently begun to be appreciated, with advances of *in vitro* models and tissue engineering (Russel et al., 1998; Wells, 2008). Matrix mechanical cues are now recognized among the key regulators in determining cellular function and behavior (Discher et al., 2005; Holle et al., 2018; Kumar et al., 2017). Indeed, substrate mechanical properties, specifically Young's modulus, have been implicated in forms of mechanotransduction, with effects on cellular responses ranging from cell motility, durotaxis, differentiation, and tumor metastasis (Engler et al., 2006; Hadden et al., 2017; Schlueter et al., 2006; Wang et al., 2012; Wong et al., 2003). Many current research efforts aim to identify elastic moduli of engineered *in vitro* microenvironments, where various normal and pathological cellular phenomena are observed, as well as their corresponding native tissue targets (Huebsch et al., 2010; Wei et al., 2015; Wijesinghe et al., 2017; Zustiak et al., 2016).

Native biological tissue has been characterized by a host of testing techniques, revealing matrix stiffness values ranging from ~17 Pa (soft tissues) to upwards of ~25 GPa (hard tissues) (Turner et al., 1999; Wellman et al., 1999), and the characterization modality utilized is often tissue-dependent. For example, tensiometry is widely used for tendons, ligaments, and muscle, whereas compression, rheometry, and indentation are typically used to characterize cartilage and other soft tissues, and nanoindentation is most often employed for bone (Levental et al., 2007). Because gels, (e.g., PDMS, agarose), are used in a variety of *in vitro* cultures and tissue engineering applications aimed to mimic or replace these tissues, they are likewise characterized by various modalities, often determined by their availability and target tissue/application. However, it is not well documented how these techniques compare in measured moduli, even within standard gels. This lack of consistency in materials characterization limits the utility of such measurements, as it does not allow moduli obtained from different testing modalities to be readily compared. Furthermore, it is likely a key contributing factor to the difficulty in replicating results of other studies, as well as the material property differences observed within the literature.

Within these modalities, there are significant differences in test sample preparation, experimental procedure, underlying assumptions, and data analysis methods. These may produce artefacts or discrepancies in calculated moduli between the different techniques. For example, tensile and compression testing involve forces applied over an entire sample, while indentation focuses loading at a single location in the material. The strain levels utilized in these techniques can also be quite different; nanoindentation and indentation are limited to small deformations (*i.e.*, nano- to micro-scale (nanoindentation), micro- to meso-scale (indentation)) typically restricted to the specimen's linearly elastic range of response (Ahearne et al., 2008; Russel et al., 1997). Rheometry also operates best at small strains (*e.g.*, 0.001 – 0.01). Conversely, compressive and tensile characterization typically measure moduli over a range of linear elastic behavior in response to large, macroscopic strains (*e.g.*,

0.05 – 0.10 in compression, 0.10 – 0.20 in tension), which may proceed until material failure.

The difference in measured properties can also be exacerbated by material viscoelasticity, which is present in many biomaterials, *e.g.*, gels and biphasic scaffold constructs. Measured moduli may vary based on differences in how the material's elastic and viscous components respond to the type of applied loading (Gupta et al., 2007). Additionally, material isotropy is needed to compare properties determined by different loading directions. While typical of many polymer gels, isotropy is not common in other biomaterials, as there is often a dominant fiber or polymer direction, similar to the directionality observed in biologic tissue. For these reasons, it is likely that even within homogenous materials, there are significant differences of measured mechanical properties across testing platforms.

The relevance of different testing modalities to the forces experienced by a cell is unclear, and widely debated; some favor indentation testing as a measure of what cells sense at focal adhesions with the extracellular matrix (ECM), others lean more toward tensiometry as a measure of the resistance a cell would experience when pulling on the material (*e.g.*, 2D substrate), and still others view bulk compression testing as a more relevant measure for 3D cellular microenvironments. As such, an improved understanding of how material properties compare when obtained by each method may prove essential for comparative analyses in the literature. Additionally, this may help to gain insight into the mechanical microenvironment experienced by cells in various 2D and 3D biomaterial applications, as well as in healthy and pathologic biologic tissues.

Herein, we compare the elastic moduli of biomaterials determined by a variety of standard characterization techniques. Agarose and PDMS gels were specifically studied because of their vastly tunable mechanical properties and their wide use in tissue engineering and *in vitro* cell-based mechanosensing applications (Cao et al., 2009; Martin et al., 2008; Palchesko et al., 2012; Wen et al., 2015). To probe whether elastic moduli were characterization-modality specific, we created cohorts of agarose and PDMS gel samples, each fabricated to cover a spectrum of modulus values (ranging from physiological to pathological/supraphysiological). We characterized each using five different modalities: tensiometry, unconfined compression, rheometry, indentation, and nanoindentation. Within all the testing techniques, the same basic trends of modulus changes were observed across the different gel fabrication parameters. However there were significant differences within their respective values and overall sensitivities.

2. Methods

2.1. PDMS preparation

Sylgard 184 (Dow Corning) polydimethylsiloxane (PDMS) was provided in a two-part kit containing monomer and a crosslinking agent. Gels were prepared in crosslinker: monomer weight ratios of 1:5, 1:7.5, 1:10, 1:20, 1:40, and 1:60. The monomer and crosslinking agent were combined to a total weight of 15 g for each group, and stirred for 2–3 min. Each gel solution was then poured into a 90-mm Petri dish, resulting in a gel approximately 2-mm

thick. The gel solutions were degassed in a vacuum desiccator until no bubbles were visible, for approximately 15–20 min, then cured at 65 °C for 12–16 h.

2.2. Agarose preparation

UltraPure Agarose (Invitrogen) was prepared at 6% (w/v) in deionized water. The solution was heated on a hot plate at 175 °C for 1 h, mixed until appearing homogeneous, then serially diluted in dH₂O to produce final concentrations of 6%, 3% and 1.5% (w/v). Gels were then poured into 90-mm Petri dishes to final volumes of 15 mL, covered and sealed to prevent dehydration, and allowed to cure for 1 h at room temperature. All characterization testing was performed on the same day as gel preparation to minimize the effects of dehydration.

2.3. General assumptions

For all testing performed, samples were assumed to be isotropic, with flat faces. Compression and indentation testing assumed that contact of the platen (or indenter) occurred normal to the specimen surface, with minimal frictional effects. Rheometric characterization made the assumptions associated with small-amplitude oscillatory shear (*i.e.*, flow is one dimensional with tangential velocities, inertial effects are negligible, stress response is linear). Last, it was assumed that minimal dehydration occurred during testing of agarose samples.

2.4. Compression testing

Cylindrical test specimens were punched from the prepared gels, using a 4-mm biopsy punch, to approximate the ratio of sample geometries (4-mm diameter, 2-mm thickness) specified by ASTM standard D575–91. Samples were characterized on a Mach-1 mechanical tester (Biomomentum Inc., Laval, QC, Canada) equipped with either a 70-N or 1.5-N load cell, depending on sample rigidity. Each test specimen was compressed to approximately 0.50 strain (absolute compression distance of 1.125 mm) at a constant rate of 0.03 mm/s. A stress-strain curve was constructed based on the measured force and displacement data (normalized to initial sample cross-sectional area and length, respectively). The elastic modulus was estimated from the linear region of this curve, which occurred between 0.05 and 0.25 strain for PDMS, and 0–0.10 strain for agarose. Six material test specimens were punched from three independently made gels, for all PDMS and agarose groups ($n = 18$ per group).

2.5. Tensiometry

Tensile testing samples were cut to a dogbone geometry, sized to ASTM standard D412–16 (gauge length = 30 mm), using a half-scale type D geometry. To enable digital image correlation (DIC), a high-contrast, anisotropic speckle pattern was applied to the test samples using the random off-axis mist of a spray paint jet (Rust-oleumVR Universal Paint & Primer-in-One, Vernon Hills, IL), creating distinct speckles of varying sizes. Each individual test specimen was then secured in an oak-tag I-frame, to facilitate gripping and to prevent damage or distortion before testing (Mubyana et al., 2016). Supplemental cardboard spacers were used with the more compliant samples (*i.e.*, PDMS 1:40 and 1:60 groups, and

all agarose groups) to prevent damage from the pneumatic grips when securing the specimen in the testing system. Initial specimen dimensions (width, thickness, and gauge length) were measured using a digital caliper, then samples were secured in a vertical Universal Testing machine (Model 500LE2–1, TestResources, Shakopee, MN), equipped with a 500 g load cell for all agarose groups and PDMS 1:40 and 1:60 groups, or a 110 N (WF12) load cell for all other samples. The supporting I-frame was then transected, leaving only the material test sample spanning grip-to-grip.

To mechanically characterize, each sample was elongated to failure at a constant rate (0.5 mm/s), while force, grip-to-grip displacement, and time data were acquired at 50 Hz. A two-camera digital image correlation (DIC) system (Correlated Solutions, Inc., Irmo, SC) was used to collect virtual extensometer data at an acquisition interval of 20 ms, and VIC Snap and VIC 2D software (Correlated Solutions) were used for image capture and analysis, respectively (Chaudhary et al., 2016; Mubyana et al., 2016). Raw force-displacement data were filtered using a 3.5 Hz lowpass filter to remove periodic noise. Filtered force and displacement data were normalized by original specimen cross-sectional area and gauge length, respectively, to convert to values of engineering stress and engineering strain. The resulting stress-strain curves were analyzed to compute Young's elastic modulus, peak stress, failure strain, and toughness (energy to the point of failure). To calculate moduli from the stress-strain curves, the slope was determined from a linear fit over a respective portion of the linear curves, (typically 10–20% of failure strain) similar to previously published work (Brown et al., 2005; Normand et al., 2000). Toughness was calculated as the area under the stress-strain curve, from the onset of strain to the point of failure, using trapezoidal integration (Corr et al., 2009; Mubyana et al., 2016). Data from the virtual extensometer, specifically the peak strain and linear strain region, were compared to the grip-to-grip displacement data. Each reported measurement was the mean of nine tests (three independently fabricated bulk gels with three test specimens produced from each) $\pm$ one standard deviation.

2.6. Rheometry

PDMS and agarose gel samples were rheologically characterized by both strain and frequency sweeps, using an AR-G2 rheometer with an 8-mm SMART-SWAP flat plate geometry (TA Instruments, New Castle, DE). The parameters for both the strain sweep and a frequency sweeps are listed in Table 1.

Preliminary sweeps were conducted on independent samples for each test group to identify the boundaries of the linear region. The preliminary strain sweep determined the constant strain value used in further frequency sweeps, and preliminary frequency sweep determined the constant frequency used in future strain sweeps. Similar to compression sample preparation, test samples were prepared by removing an 8 mm-cylindrical punch from the material Petri dish and measuring the thickness with a digital micrometer (Marathon Management Co.). Based on the micrometer measurements, the gap distance was set to achieve a 1% strain on the sample, to prevent sample slipping. Three independent gels were fabricated per condition, and three samples were punched per gel ($n = 9$). Each sample was

used for only one test, and the results indicate the mean and standard deviation of the nine trials.

The elastic modulus was estimated from rheometry data within the linear-viscoelastic regime, as previously described (Zuidema et al., 2013), using the assumption of small-amplitude oscillatory shear (SAOS). The equilibrium shear modulus was estimated as the plateau value of the storage modulus (G') at low frequencies, obtained from the frequency sweep data. Data taken at 1 Hz and below were used in calculation of the modulus for both materials. The elastic modulus (E) was calculated based on the equilibrium shear modulus (G) using Eq. (1), where ν represents Poisson's ratio, estimated to be 0.5 for both PDMS and agarose, for the strains used throughout this work (Johnston et al., 2014; Normand et al., 2000; Wang and Krause, 1987)

$$E = G(1 + \nu), \quad (1)$$

2.7. Indentation testing

Indentation characterization was performed on samples within 90-mm Petri dishes, using a Mach-1 mechanical tester (Biomomentum Inc., Laval, QC, Canada) with a spherical tip probe (6.35 mm diameter). A 1.5-N or 70-N load cell was used for all samples, depending on sample rigidity. To characterize, the probe height was first calibrated to a Petri dish containing no gel, to standardize the height of the probe. Gel tests began using the system's "Find Contact" mode, wherein the probe is lowered at a rate of 0.03 mm/s until a load was detected. Based on the determined initial sample height, the test was set to a total probe displacement of 30% strain, at a constant rate of 0.03 mm/s for all samples. The elastic moduli of indentation data were estimated using a Hertzian model, which accounts for the contact mechanics between a sphere and an elastic solid (Dyson et al., 2017; Li et al., 2006). To utilize the Hertz model, measured indenter force was plotted against the indentation depth raised to the 3/2 power, and the slope of the initial linear region, (between 0 and 0.1 strain for PDMS, and 0.03–0.09 strain for agarose), was used to solve for elastic modulus, E , in Eq. (2),

$$F = \frac{4}{3}ER^{1/2}d^{3/2}\frac{1}{(1 + \nu)}, \quad (2)$$

where, F is detected force, R is the radius of the indenter, d is the displacement, and ν is the Poisson's ratio of the sample. Three independent gels were fabricated for testing per sample condition with six indentations per gel ($n = 18$).

2.8. Nanoindentation testing

Nanoindentation experiments were performed with a Hysitron TriboIndenter (Hysitron Inc., Minneapolis, MN) in a load-controlled instrument with a load resolution of 100 nN, displacement resolution of 1 nm, and maximum indentation depth of 5 μm . Previous studies reported that indentation size has a pronounced effect on measured modulus and hardness values in soft polymers when a standard 100-nm curvature Berkovich tip was used, and that

using a spherical tip would yield consistent values for modulus and hardness, regardless of the indentation depth (Alisafaei and Han, 2015; Han et al., 2016). Thus, for all experiments, a hemispherical indenter of radius of 831 nm (TI-0043, Hysitron) was used. We performed a series of nanoindentation tests on a fused silica standard (S/N 5–0098, Hysitron) to obtain the contact area function parameters, as instructed by the manufacturer. Nanoindentation experiments were performed at room temperature as previously described (Carrillo et al., 2005). For PDMS samples, quasi-static nanoindentation testing was performed with a trapezoidal load *versus* time profile, with a loading and unloading rate of 4 $\mu\text{N/s}$, a peak of 40 μN , and a hold period of 10 s (30 s total time profile). For PDMS experiments, each sample was tested with 25 indents (5×5 grid with 5 μm separation). For agarose samples, controlled displacements of 3000 nm were used with the same trapezoidal load vs time profile, each sample was tested with nine indents (3×3 grid with 5- μm separation). Because soft tissues and hydrogels have a significant water content, and their mechanical properties can change significantly with swelling/ contraction, it is necessary to study soft polymers in a hydrated state to obtain consistent results (Kaufman et al., 2008). Each agarose gel sample was placed in the center region of a surrounding saline-hydrated sponge ring, to maintain sample hydration during characterization, as previously described (Ebstein et al., 2001). To measure the reduced modulus (E_r), a curve was fit to the unloading portion of the load *versus* time response. The elastic modulus (E) was then calculated through the relationship:

$$\frac{1}{E_r} = \frac{1 - \nu_i^2}{E_i} + \frac{1 - \nu_s^2}{E_s}, \quad (3)$$

where ν_i and ν_s are the Poisson's ratio of the diamond spherical-tip indenter (0.07) and substrate material, respectively, and E_i is the modulus of the indenter (1140 GPa) as provided by the manufacturer (Oliver et al., 1992).

2.9. Statistical analyses

A one-way ANOVA was used to examine if mean elastic modulus values for each experimental group were different based on the characterization method used. Then, a multiple comparison test was used to determine which testing methods produced significantly different means, if any. All statistical analyses were performed in MATLAB (version R2016b, MathWorks, Inc.), using the “anova1” and “mult-compare” built-in functions, with $p < 0.05$ for statistical significance.

3. Results

3.1. Compression testing

Compression testing of PDMS gels produced characteristic stress-strain curves (Fig. 1a), which became linear within approximately 5% strain, and nonlinear at strains greater than approximately 25%. PDMS moduli followed a trend with respect to crosslinker:monomer ratio, with values increasing in order of 1:60, 1:40, 1:20, 1:5, 1:7.5 and 1:10. Stiffness (moduli) increased with decreasing ratio of crosslinker to monomer until 1:10 ratio was

reached, after which the modulus began to decrease (Fig. 1b). Elastic moduli for these gels ranged over three orders of magnitude, from approximately 20 kPa (1:60) to 3.2 MPa (1:10).

Agarose gels also displayed characteristic stress-strain curves, (Fig. 1c), with an immediate linear elastic response, that remained constant until ~10–15% strain. Elastic moduli increased with increasing agarose concentration, over the 1.5–6.0% range tested, with values ranging from approximately 36 to 555 kPa (Fig. 1d).

3.2. Tensile testing

Representative stress-strain curves in PDMS (Fig. 2a) show that although both strain measurement techniques are able to appreciate the strain-stiffening behavior of PDMS, the grip-to-grip measurement overestimates the strains experienced within the specimen gauge length, leading to a longer linear region with a less steep slope. Thus, grip-to-grip values appear to underestimate the true material modulus and overestimate sample extensibility. Despite this discrepancy, for analysis and comparison between other testing techniques, grip-to-grip (TestResources™) strain data were used for all calculations and comparisons between characterization methods. These values are directly measured by the testing apparatus, not within the sample itself, making them analogous to the other testing modalities explored. Within PDMS groups, modulus and peak stress followed the same trend, with values increasing, in order, 1:60, 1:40, 1:20, 1:5, 1:7.5 and 1:10; exhibiting the same trend as observed in PDMS compression testing (Fig. 2b,c). Similar to compression testing, PDMS tensile moduli spanned three orders of magnitude, from approximately 12 kPa (1:60) to 2.0 MPa (1:10). Conversely, peak strain decreased with crosslinker to monomer ratio, until a 1:10 ratio was reached, after which there were no further changes in extensibility (Fig. 2d).

Similar to PDMS, the grip-to-grip measurement of agarose gels overestimates the strains experienced within the specimen gauge length (Fig. 2e), leading to lower moduli and larger failure strains than would be expected from extensometer-based measurements. Agarose gels' elastic moduli ranged from 50 kPa to 587 kPa, and increased with matrix content, as expected (Fig. 2f). A similar trend was seen in peak stress (Fig. 2g), consistent with the results of agarose compression testing. In addition, agarose gels were significantly more brittle than PDMS gels, failing at strains below 30% compared to PDMS which often experienced strains over 100% prior to failure (Fig. 2h).

3.3. Rheometry

Rheometric characterization produced measures of storage modulus (G'), loss modulus (G''), and complex modulus (G^*) for each experimental group (Table 2). The preliminary frequency and strain sweeps for each material group, PDMS (Fig. 3b,c) and agarose (Fig. 3e,f), revealed artefacts at high frequencies and strain values, due to either inertial effects from the test (*i.e.*, inertial forces are greater than material stiffness), structural changes in the sample, or slippage within the test. These preliminary test data were used to establish the appropriate operating ranges for strain (0–0.05 and 0–0.002 in PDMS and agarose, respectively) within the frequency sweep (0–1.0 Hz) for subsequent material specimen characterization. Elastic moduli determined *via* rheometry, summarized for PDMS (Fig. 3a)

and agarose (Fig. 3d), exhibited strikingly similar trends with respect to gel composition, to those of compression and tensile testing, as well as similar absolute modulus values.

3.4. Indentation testing

Force–(displacement)^{3/2} curves, representative of all samples for PDMS (Fig. 4a) and agarose (Fig. 4c), were used to calculate elastic moduli for both materials, summarized in Fig. 4(b,d), which displayed data trends consistent with the aforementioned testing modalities. Namely, the PDMS gels exhibited significant changes in moduli with crosslinker:monomer ratios, with ratios in ascending order of elastic moduli 1:60, 1:40, 1:20, 1:5, 1:7.5 and 1:10; and agarose gel moduli increased with increasing agarose concentration. In PDMS, moduli measured *via* indentation appeared to be similar to compression and rheological values, and greater than those determined by tensiometry. In agarose gels, indentation-derived moduli were larger than those found by compression, tensile, or rheologic characterization; and this difference was more pronounced with increasing matrix content.

3.5. Nanoindentation testing

Nanoindentation was used to compare the effects of probe size and displacement (micro *vs.* nano), on the measured modulus. PDMS gels exhibited a characteristic loading and unloading curve (Fig. 5a), similar to previous work, with differences in force magnitude likely due to the size of the indenter (Wang et al., 2014). Reduced modulus, hardness, and elastic modulus from nanoindentation (Fig. 5b) all reflected similar trends with crosslinker:monomer ratio, to those observed using the macro-scale characterization modalities, herein. However, while elastic moduli followed the similar trends, they were larger than those of the other modalities (*i.e.*, tensiometry, compression, rheometry, indentation). In the agarose gels, nanoindentation produced characteristic loading and unloading curves (Fig. 5c), similar to previous observations (Ebenstein and Pruitt, 2004). As with PDMS, the trends observed in the agarose gels were consistent with those of the macro-scale characterization modalities. Agarose moduli increased with weight percentage (Fig. 5d), and nanoindentation measured greater elastic moduli than the macro-scale modalities.

3.6. Comparison of methods

Direct comparisons of moduli between the different characterization modalities, for both PDMS and agarose gels, are presented in Fig. 6a,b, respectively. A one-way ANOVA revealed a difference due to characterization modality, and pair-wise comparisons between the different modalities determined the source of these observed differences.

Nanoindentation consistently measured higher moduli than all other techniques, for all PDMS and agarose samples. This is clearly shown in the ratios of measured elastic moduli between different characterization modalities, in both PDMS and agarose gels (Table 3). In the PDMS samples, tensiometry produced the lowest elastic moduli, with fairly consistent values between compression, indentation, and rheometry. In agarose, the moduli were comparable for all modalities, other than nanoindentation, but at the highest agarose concentration (6%) indentation testing determined a significantly larger modulus value.

To further compare these characterization modalities, a power curve was fitted to the modulus *vs.* monomer/crosslinker (PDMS) curve, and the modulus *vs.* concentration (agarose) curve, to identify the sensitivity of each modality to stiffness changes in PDMS and agarose, respectively. In this analysis, greater power coefficients indicate a greater sensitivity to changes in material modulus (Table 4). For agarose, compression testing was most sensitive to changes in modulus, followed by rheometry, indentation, and tensiometry, with nanoindentation far less sensitive than the rest. For PDMS, rheometry proved to be the most sensitive technique, followed by tensiometry, compression, indentation, and lastly nanoindentation.

4. Discussion

Within this study, we employed five commonly used mechanical characterization modalities on cohorts of PDMS and agarose gels engineered to span a range of elastic moduli, from physiological to super physiological values (kPa to MPa), to explore whether the measured elastic moduli were influenced by the testing method. For agarose samples, each of the different modalities consistently determined the 6% group to be the stiffest, and 1.5% group as softest; a trend expected based on the concentration of the polymer component in the gel. For all PDMS testing, the overall trend in modulus was more complex, but was similarly well-defined within all testing modalities; the order of gels (crosslinker:monomer ratio) from stiffest to most compliant was consistently 1:10, 1:7.5, 1:5, 1:20, 1:40 and 1:60. While our experiments showed an expected increase in gel stiffness with increasing crosslinker concentration, up to a crosslinker:monomer ratio of 1:10, there appeared to be an inflection at this point, beyond which the gel became more compliant with increasing crosslinker concentration (*i.e.*, 1:7.5 and 1:5 groups). Moreover, this trend was observed in all five testing modalities explored herein, and is supported by observations from several previous PDMS studies, and is thought to be attributed to the presence of mobile and unbound crosslinker molecules beyond the crosslinking saturation point of PDMS, at approximately a 1:10 wt ratio (Khanafer et al., 2009; Lee et al., 2004; Seo et al., 2013). The replication of the expected modulus trends, in both gel cohorts, across all testing methods, suggests that each characterization technique is sensitive to the overall trends in material behavior.

While each testing modality was able to capture the trends in material behavior for both agarose and PDMS, the different modalities did not determine identical elastic moduli for the same material, suggesting that their absolute values for modulus are characterization modality-specific. This is not particularly surprising, since each modality has unique assumptions, and operates over distinct strain regimes. The bulk material testing modalities (*i.e.*, rheometry, compression, and tensiometry) all assume a uniform strain field to calculate modulus, which is not a constraint of indentation techniques. For characterizing soft hydrogels, *e.g.*, PDMS and agarose, these bulk testing modalities offer unique advantages and distinct challenges.

Rheometry is able to monitor changing gel properties (*e.g.*, during hydrogel gelation), however it is susceptible to artefacts at high frequencies and/or large amplitudes of applied strain, as seen in our pilot data. Such loadings can cause inertial effects within the gel, slip between the platen and sample, and possibly damage the sample, leading to erroneous

underestimation of modulus, which is why it is critical to establish the linear elastic regime for characterization. Conversely, tensiometry allows for large, macroscopic strains and failure testing of soft gels, enabling the characterization of fracture and toughness values. However, soft gel tensiometry can be complicated by non-uniform strain fields, and difficulties in gripping the specimen. To address these challenges, we utilized an ASTM-standard dogbone specimen geometry to achieve uniform strains throughout the sample gauge length, and validated these with concurrent digital image correlation (DIC) analyses (data not shown). Gripping soft gel specimens is a delicate balance: the gripping must be sufficiently strong to prevent specimen slip within the grips, yet gentle enough to avoid damaging the specimen or developing stress concentrations at the grip – either of which would lead to underpredictions of elastic moduli. Herein, we employed I-frame specimen holders, with cardboard spacers, to provide sufficient gel gripping without damaging the specimen; there was no evidence of either slip (between grip and I-frame, or gel within frame) or stress concentrations at the grip. This was further supported by sample failures occurring in mid-substance (within the gauge length) for all tensile tests. While this proved to be very effective for all gel formulations studied herein, there will be a limit to how soft a material can be for tensile characterization, which is not a limiting factor in the other techniques explored.

Nanoindentation and AFM-based techniques measure the properties at the point of load application to gain a local material property measurement. This provides the unique ability to measure material property heterogeneity across a specimen surface. However, this can also lead to a range of values for a given material, due to regional or structural heterogeneity in the sample. This variation is expected to increase as the probe size decreases, such that the largest error bars are associated with nanoindentation. Surprisingly, this was not the case in our study, and moduli from indentation tests were no more variable than those of the other, bulk-scale characterization modalities. However, these techniques have limitations such as error introduced with testing depths much greater than the radius of the probe head, nonlinearity, adhesive samples (specifically PDMS), potential differences in the surface structure compared to the bulk of the material, and the surface interactions - much of which is seen as an artefact amplified by the size of the nanoindenter (Han et al., 2016). For soft polymers, adhesion effects may be observed during nanoindentation experiments, which results in overestimation of the modulus (an observation repeated herein). This phenomenon, zero load plastic deformation, occurs when the indenter tip is pulled away from the sample. The adhesive attraction deforms the soft polymer along the direction of the tip motion and causes a negative indentation. However, the attraction forces between the tip and the surface can be minimized by accurately determining the zero displacement position (Charitidis, 2011). Performing the measurements on a hydrated samples significantly lowers the attraction forces between the PDMS and tip (Carrillo et al., 2005). In addition, the adhesion effect is more pronounced when a sharp tip (standard Berkovich tips with a curvature of 100 nm) is used and displacements are less than 1000 nm (Charitidis, 2011). For nanoindentation experiments conducted in this study, the adhesion effect was minimized by hydrating samples, using large spherical tips, and keeping the displacement greater than 1000 nm. Although greatly minimized by our experimental approach, these adhesion effects may, in

part, explain the larger moduli measured with nanoindentation, which would be an interesting topic for future experiments.

The strain levels utilized in these techniques can also be quite different, and as a result, the materials may be characterized at varying degrees of structural deformation. In general, when subjected to uniaxial loading (tension, compression, or shear), soft hydrogels, like most polymer networks, typically exhibit three strain regimes: an initial linear elastic response at small strains, followed by a non-linear regime (strain-stiffening in tension and shear, and strain-softening in compression) at intermediate strains, and a second linear regime at larger strains. In the first strain regime, the material response is linearly elastic, little structural change occurs, and the measured modulus is primarily strain-independent. In the second and third regimes, structural deformations occur based on loading conditions (*i.e.*, strain-stiffening, strain-softening) and the instantaneous modulus of the material is influenced by the strain state. As a result, different modalities would be expected to yield similar modulus values when characterization is constrained within the first regime, due to the small structural deformations and relative strain insensitivity of moduli, and these values are expected to diverge when characterization extends into second or third regimes. These behaviors are supported by elegant prior theoretical modeling studies utilizing random fiber networks; appropriate models for both PDMS and agarose. When characterized within the first (linear elastic) strain regime, fiber network models predict identical effective moduli in both tension and compression, and these values diverge when characterized over greater strain ranges because of structural changes produced within and after the nonlinear strain regime (Dillen et al., 2008).

Within our experiments, moduli differences between testing modalities were more pronounced at greater gel stiffnesses. However, within the less extensible agarose gels, tensile, compression, and rheological characterization resulted in similar moduli, implying that any of these three techniques are suitable equivalents and allow values to be directly compared for agarose testing, within the ranges explored. This is likely due to the material exhibiting a linearly elastic stress-strain response over the strain ranges used for characterization. Such similarities in moduli are predicted by fiber network models, when characterization is performed with limited structural deformations in the linear elastic strain regime (Islam and Picu, 2018). While the stiffest PDMS formulations (1:10, 1:7.5, 1:5) displayed similar behavior, the PDMS samples showed diverging moduli as the gels became more compliant, with tension and shear (rheometry) predicting significantly lower modulus values than those found *via* compression. This may be due to a later emergence of linear stress-strain regions in the more extensible gel. Mechanistically, this difference could be associated with structural evolutions that occur within the gel network during the non-linear strain regime of testing (Statics, 2005; Tharmann et al., 2007).

Indentation produced similar values at the lower agarose concentrations, but significantly larger elastic moduli than these three modalities for the stiffer, 6% agarose gels. In PDMS, compression and indentation testing yielded similar moduli for each of the gel ratios in the cohort, however, rheometry appeared to produce slightly lower moduli in some sample groups. Conversely, tensiometry-determined moduli were lower than those determined by all other modalities, for nearly every sample studied. The fact that these lower tensiometry-

based moduli were observed in PDMS, but not in agarose, may be due, at least in part, to the large strains that PDMS undergoes prior to failure (~100% or greater) compared to agarose during failure testing. Such large PDMS strains create a greater discrepancy between the range of strains used to determine moduli *via* tensiometry, and those used in the other characterization modalities. Moreover, PDMS samples required a longer testing duration to achieve these large strains, more than triple the test duration of agarose, which may allow greater energy dissipation (*e.g.*, stress relaxation) during the test, due to the gel's viscoelastic properties. The largest overall differences in reported moduli were observed between nanoindentation testing, which was significantly greater in every test group than the four other characterization modalities. This is consistent with work that compared select PDMS formulation mechanical properties between compression and nanoindentation (Carrillo et al., 2005). Our measured difference was most pronounced within agarose samples, and may be due to the size of probe used, which has been identified to overestimate modulus inversely proportional to probe size (Crichton et al., 2013). In addition, the difference could also be an artefact of material heterogeneities affecting the stiffness at nanoscale testing depths, an effect not present in macroscopic testing methods because such heterogeneities are normalized over the bulk of the material during characterization. This is, at least in part, supported by literature which identified that within PDMS gels there was a higher crosslinking density at the surface than in the interior of the gel (Wrucke et al., 2013).

Together, these data highlight the potential for error in direct comparison of mechanical properties measured using different material characterization techniques. Our results suggest that the degree of moduli similarity between the different macroscopic testing modalities may vary based on the material's compliance; with a generally better agreement between modalities observed in the softer samples; *e.g.*, the three agarose groups and the softer PDMS (1:60, 1:40) groups, all of which have moduli in the kPa range. Modulus similarity between the different characterization methods may also be material-specific. Microstructure may significantly impact how loads are distributed through a material, determining the response of a substrate to applied force (Mullen et al., 2015). Therefore, caution should be used when attempting to extrapolate these observations in PDMS and agarose gels to other gels, biomedical materials, and biologic tissues; particularly those with different microstructures. This is especially true for materials that exhibit nonlinear, anisotropic viscoelastic mechanical responses, as these behaviors are expected to exacerbate the observed discrepancies in moduli between different characterization modalities. For example, fibrous materials and tissues will likely exhibit different mechanical properties in tension, compression, and shear, based on fiber density, distribution, crosslinking, and orientation with respect to the direction of loading. This has been observed in collagen-based structures, where different modes of testing determined moduli that were orders of magnitude different within the same sample (Almaraz and Athanasiou, 2004).

Based upon these experimental results, as well as other published findings and fiber network modeling studies, we make the following recommendations for comparing moduli obtained from different testing modalities. Because the modulus values can be significantly influenced by the modality employed, as well as the strain regime(s) utilized in characterization, it is important that these parameters are clearly stipulated when providing moduli for any biomaterial, to enable comparisons to published values. For isotropic

polymeric gels or biomaterials, that do not exhibit directional bias or principal fiber orientations, and can be effectively modeled as random polymer networks (*e.g.*, agarose, PDMS), reliable direct comparisons can be made between different macroscopic bulk specimen testing modalities (*e.g.*, tension, compression, and rheometry), provided characterization is performed in the first, linear-elastic strain regime. Thus, any of these bulk macroscopic techniques can be viewed as analogous in small-strain regimes, and the specific modality for characterizing a new biomaterial can be chosen based on sample geometry and intended application, as appropriate. Micro- and nano-indentation provide powerful means to quantitatively compare properties of different materials or material formulations, and are particularly well-suited for intra-study comparisons. However, these indentation techniques are susceptible to additional potentially-confounding effects (*e.g.*, surface interactions, probe size), and great care must be taken when extrapolating their results for direct comparison to other studies. Lastly, while small-strain macroscopic characterization enables direct comparisons between modalities, based on our findings, it is strongly recommended that polymeric gels and biomaterials are also characterized utilizing the strain regime(s) and mode of loading (*e.g.*, tension, compression, shear) they are likely to experience in their designed application, since these can greatly affect material performance beyond the small-strain regime.

There are several important follow-up studies that will be key to: (i) reveal the source(s) of these observed characterization technique-dependent differences in measured properties, and if possible, (ii) normalize or calibrate the properties measured between different mechanical testing modalities, to allow comparative analyses between disparate characterization methods. An important observation in this work is the difference in mechanical properties between indentation-based techniques, and bulk testing methods. Indentation-derived moduli were slightly larger than those found at the bulk material level (*i.e.*, tension, compression, rheometry), and this difference was magnified when performed with a nano-scale probe (*i.e.*, nanoindentation). Together, these findings suggest a difference in surface *vs.* bulk material properties, that is greatly influenced by scale. Therefore, future work will aim to compare the mechanical and structural properties throughout a bulk gel (*i.e.*, surface and interior), to determine if there is regional heterogeneity in moduli, and if it correlates to differences in material microstructure. This can be augmented by a theoretical modeling approach, to determine if gel microstructural differences, throughout and between the materials, can account for some of the difference(s) observed between these techniques.

5. Conclusions

Overall, five different modalities, commonly used to mechanically characterize biomedical materials, showed similar elastic modulus trends in both PDMS and agarose gels, supporting the ability of each to quantify relative changes in modulus, over a range spanning from physiologic to pathologic (supra-physiologic) values. However, these testing modalities can yield significantly different elastic moduli for the same material. The extent of these differences may be related to the material's compliance and microstructure, and depends on the testing methods being compared. As a result, in many cases, the "material properties" measured using different testing methods are not directly comparable, but are modality-specific, and thus cannot be directly compared when obtained using disparate

characterization modalities. These findings underscore the need for well-defined methods for robust, standardized characterization, to enable direct comparison of biomedical material properties between research laboratories, regardless of the modality employed. Future work can probe materials spanning a larger range of stiffnesses, and with more varied microstructures, to identify how these material characteristics influence moduli obtained by the different characterization modalities. With this improved understanding, we aim to model the trends of testing modalities to identify potential means of comparison and prediction between characterization modalities.

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Nomenclature

E	Elastic modulus
G	Shear modulus
TR	Tensile testing data with strain data measured by the tensile testing machine (TestResources™)
Ext	Tensile testing data with virtual extensometer strain data, measured using DIC
ν	Poisson's ratio

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Fig. 1. Characteristic compression stress-strain curves and calculated elastic modulus values for each testing group of (a, b) PDMS ($n = 18$) and (c, d) agarose ($n = 18$). Elastic moduli values were determined using linear strain regions defined as between 0.05 and 0.25 for PDMS, and 0 and 0.1 strain for agarose.

Fig. 2.

Characteristic tensile stress-strain curves, and resulting elastic moduli for each experimental group of (a, b) PDMS ($n = 9$) and (e, f) agarose ($n = 9$). “TR” denotes grip-to-grip strain data calculated by the Test Resources universal testing machine, and “Ext” indicates strain calculated within the sample gauge length using a DIC-based virtual extensometer. Peak stress (c, g) and failure strain (d, h) values are also provided for each of the polymers.

Fig. 3.

Elastic moduli calculated using the G' values for (a) PDMS and (d) agarose at low frequencies (less than 1 Hz). (b) Storage Modulus (G') results from small amplitude shear stress (SAOS) frequency sweep (0.01–100 Hz) of the PDMS samples. (c) Storage Modulus (G') results from SAOS strain sweep (0.001–1) of the PDMS samples. (e) Storage modulus (G') results from SAOS frequency sweep (0.01–100 Hz) of the agarose samples. (f) Storage Modulus (G') results from SAOS strain sweep (0.001–1) of the agarose samples. Values are the mean of 3 replicates from 3 independent samples for each group ($n = 9$).

Fig. 4.

Characteristic force – displacement^{3/2} curves and average calculated elastic modulus values for each testing group of (a, b) PDMS and (c, d) agarose. Elastic moduli values were determined using Eq. (2) with the slope of a force – displacement^{3/2} graph within strain regions between 0 and 0.1 for PDMS, and 0.03 and 0.09 for agarose. Values are the mean of 6 replicates from 3 independent samples for each group (n = 18).

Fig. 5. Nanoindentation-based force – displacement curves from PDMS samples (a) and agarose (c). Values presented are the mean of 25 and 9 indentations from 3 independent samples for PDMS (b) and agarose (d) respectively ($n = 75$ and 27).

Fig. 6.

A comparison of elastic moduli determined by the different characterization modalities, for (a) the six different experimental groups of PDMS, and (a) the three different agarose concentrations. Bars between groups indicate a statistically significant difference in means ($p < 0.05$).

Table 1

Temperature, frequencies, and strains used for PDMS and agarose rheological testing.

	Parameter	Strain sweep	Frequency sweep
PDMS (1:5, 1:7.5, 1:10, 1:20, 1:40, 1:60)	Temperature	23.0 °C	23.0 °C
	Frequency	1.0 Hz	0.01 Hz–100.0 Hz
	Strain	0.001 – 1 (0.1%–100%)	0.05 (5%)
Agarose (1.5%, 3%, 6%)	Temperature	23.0 °C	23.0 °C
	Frequency	0.2 Hz	0.01 Hz–100.0 Hz
	Strain	0.001 – 1 (0.1%–100%)	0.002 (0.2%)

Table 2

Storage Modulus (G'), Loss Modulus (G''), and calculated Complex Modulus (G^*) values of PDMS and agarose obtained by rheometry.

	PDMS						Agarose		
	1:60	1:40	1:20	1:10	1:7.5	1:5	1.5%	3%	6%
G' (kPa)	3.7 ± 0.70	20.2 ± 0.7	290 ± 19	1037 ± 49	930 ± 2	617 ± 22	10.2 ± 0.4	41.2 ± 31.6	180 ± 53
G'' (kPa)	0.6 ± 0.05	1.7 ± 0.05	15.0 ± 1.1	41.9 ± 2.1	24.7 ± 6.1	10.2 ± 1.5	0.7 ± 0.06	4.1 ± 1.8	20.9 ± 1.9
G^* (kPa)	3.7 ± 0.69	20.2 ± 0.7	291 ± 19	1038 ± 48	931 ± 2	617 ± 22	10.3 ± 0.4	41.5 ± 31.5	181 ± 53

Table 3
Ratios of measured elastic moduli between different characterization modalities (column/row).

Modality	Compression	Tension	Rheology	Indentation	Nanoindentation
PDMS					
Compression		0.50 ± 0.18	0.73 ± 0.27	1.0 ± 0.20	2.6 ± 1.9
Tension	2.2 ± 0.90		1.4 ± 0.27	2.4 ± 1.3	7.0 ± 8.7
Rheology	1.6 ± 0.70	0.71 ± 0.15		1.7 ± 1.1	4.6 ± 5.8
Indentation	1.0 ± 0.20	0.53 ± 0.24	0.77 ± 0.37		2.5 ± 1.3
nanoindentation	0.49 ± 0.19	0.28 ± 0.15	0.42 ± 0.25	0.45 ± 0.14	
Agarose					
Compression		1.3 ± 0.02	0.74 ± 0.27	2.0 ± 0.26	8.3 ± 6.9
Tension	0.78 ± 0.14		1.1 ± 0.10	1.5 ± 0.14	6.2 ± 4.8
Rheology	0.74 ± 0.15	0.94 ± 0.09		1.5 ± 0.25	6.0 ± 5.3
Indentation	0.51 ± 0.07	0.66 ± 0.06	0.71 ± 0.13		4.0 ± 2.9
nanoindentation	0.38 ± 0.15	0.24 ± 0.15	0.26 ± 0.17	0.36 ± 0.24	

Table 4

Concentration/crosslinker-monomer ratio and modulus relationship.

Test method	Agarose coefficient	PDMS coefficient
Compression	1.98	2.81
Tension TR	1.78	3.21
Rheology	1.87	3.67
Indentation	1.79	2.53
Nanoindentation	0.98	1.67