Physical and Mechanical Properties of RAFT-Stabilised Collagen Gels For Tissue Engineering Applications.

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Abstract

Cell behavior is influenced by the mechanical and structural properties of their substrate environment. Also, materials mechanically resistant to surgical handling and similar to the host site are required in tissue engineering to minimise the chance of an adverse host response. RAFT-Stabilisation is a commercially available technique for creating stabilised hydrogels. Properties of RAFT-stabilised collagen (RsC) gels are governed by size, composition and arrangement of fibrils and their interaction with the fluid trapped within the matrix. The stabilisation process, using hydrophilic porous absorbers, produces dense matrices by rapid expulsion of fluid, and the structure obtained has mechanical properties suitable for tissue engineering. However, protocols to define and compare the physical properties and mechanical behavior of RAFT-stabilised collagen gels are not standardised across the field. Here, we investigate the fundamental mechanical and structural properties of RsC gels, and propose a new empirical relationship that correlates the measured stiffness of gels to varying frequency of strain oscillation. The results provide quantitative data characterising this extracellular environment for future tissue engineering studies.

Keywords: Collagen gel, Rheology, Dynamic Mechanical Analysis, Frequency Sweep, RAFT-stabilisation.

1 Introduction

Collagen type I is a natural polymer commonly used as a biomaterial in tissue engineering for numerous applications such as peripheral nerve regeneration[1,2], bone reconstruction[2,3], drug delivery[4,5], and skin reconstruction.[2,6] Its biodegradability, biocompatibility, high versatility and its ready availability are major advantages for application in the field of tissue engineering[7] and makes collagen type I suitable for implantation (although a very small proportion of the population is allergic to it).[8] Collagen provides both structural support and guidance cues which influence cell proliferation, differentiation, and migration when cells are cultured in/on a collagen hydrogel substrate.[3,9–15] This is key to mimicking the environment in the body, where cell interactions with the extra-cellular matrix (ECM) produce a traction-induced signal directly dependent on the mechanical constraints provided by the ECM.[2,16–18] Indeed, it is now widely accepted that the stiffness of the substrate has a direct influence on cell behavior.[19]

To mimic the natural tissue mechanical and structural properties, collagen hydrogels used for tissue engineering purposes are often blended or cross linked in order to obtain replacement or repair solutions that would complement natural repair processes[16,20,21], e.g. for wound healing and regenerative purposes.[2,6,22–25] Without modification of the fully-hydrated collagen gel structure, there tends to be a mechanical and structural disparity compared with many mature body tissues.[26–28] In 2005, Brown, et al[26], developed a process to rapidly produce dense collagen matrix through plastic compression, opening a new route for the production of materials structurally and mechanically suitable for tissue engineering. The matrix produced by plastic compression, a combination of external mechanical loading and fluid absorption, is a dense collagen structure obtained by expulsion of 97% of fluid from the hydrogel.[26] This process increases the strength and mechanical integrity of the hydrogel[26], making it mechanically more comparable to soft human tissues. As shown in Brown et al[26], under tensile testing, the ultimate tensile strength of plastic compressed gels was 0.55 MPa, approaching native tissue values. They also showed high cell viability for plastic compressed gels and compressed + tensioned gels (5-30% strain), indicating minimal impact of the fluid removal process on cell survival[29], and making it a promising technique for the biomaterial and tissue engineering field. Soon after this initial work, Neel, et al[30] showed the

importance of the level of hydration on the behavior of collagen scaffolds (hyper hydrated, single (SC) and double compressed (DC)). For these studies, plastic compression was performed with a constant load of 60g per cross-sectional area (mm^2)to induce a downward fluid flow.[26,31] The SC and DC constructs were shown to support cell seeding, their hydration level did not interfere with the cell viability and they have been used for numerous tissue engineering applications.[29]

More recently, Levis et al[31] extended the plastic compression approach to the commercially available Real Architecture For 3D Tissues (RAFT) kit, allowing confined compression (CC) of hydrogels with upward flow, known as RAFT-stabilisation, in collaboration with TAP Biosystems (**Figure** 1). This method is not experience dependent and provides a rapid, simple and consistent way to fabricate engineered tissues able to withstand handling. As this approach becomes widely adopted as a reproducible method for rapidly constructing dense sheets of cellular material, it is important to understand the structural and mechanical properties of these matrices of collagen after RAFT-stabilisation.

The mechanical characterisation of biomaterials is a well-established field and a range of different tests and techniques are commonly used (e.g compression, uniaxial tension, shear stress) to establish viscoelastic properties for isotropic and homogeneous materials.[32] As the mechanical values obtained depend on the type of stress applied, it is important to do an extensive analysis using multiple approaches.

The aim of this study was to characterise thoroughly the mechanical behaviour of RsC gels. RsC gels were fabricated using multiple initial volumes, in either 24 or 96 well-plates, using the CC RAFT process.[31] First of all, the collagen density was calculated based on measurements of the final dimensions of the gels, and variation in mechanical response of RsC gels under a sinusoidal load with frequency-dependent oscillations was reported. Next RsC matrices were tested under compressive, tensile and shear stress across a range of frequencies and their mechanical behavior was analysed and correlated to the physical properties. Finally new empirical relationships were determined which link Young's modulus to frequency for each of compressive, tensile and shear loading. These were determined based on regression analysis on the experimental data obtained for each mechanical test, and provide a useful predictor of RsC matrices for future studies.

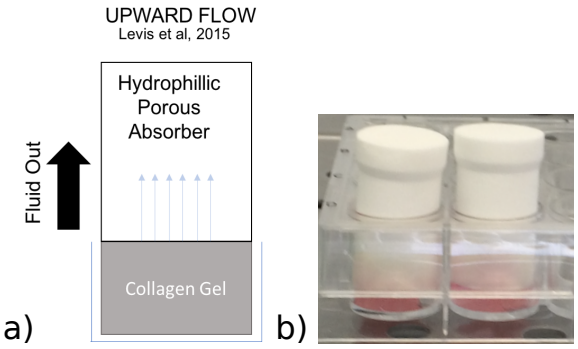


Figure 1: a) Levis et al[31], schematic of the confined compression (CC) RAFT-stabilisation process with upward flow. b) Image of collagen gels undergoing RAFT-stabilisation in 24 well-plates.

2 Materials and methods

2.1 Preparation of RAFT-stabilised collagen gels

The collagen solution was formed using acid-solubilised type I collagen solution from rat tail tendon (2 mg.mL^{-1} in 0.6% acetic acid; First Link, UK). For each collagen solution, the following components kept on ice, and their respective percentage of the final volume, were mixed, in order : 80% (v/v) rat tail collagen type I, 10% (v/v) 10×Minimum Essential Medium (MEM), 5% (v/v) Dulbecco's modified eagle's medium (DMEM), and neutralised with 5% (v/v) 0.325M sodium hydroxide to achieve physiological pH, thus inducing the collagen gelation process[15,33]. The collagen solution was pipetted into well plates ($\text{gel}_{concentration}$ = 1.64 mg.mL^{-1} and area covered per well plate being 201 mm^2 for 24 well plates and 29.6 mm^2 for 96 well plates) and kept in a humidified cell culture incubator (37°C, CO_2 = 5%) for 10 min to allow gelation. Then, gels were RAFT-stabilised for 15 min using RAFT®absorbers fitting the well plates size (96 and 24 well plates) (Startorius Stedim/Lonza). This step rapidly removes most of the fluid of the hydrogel through the top surface of the gel (Figure 1) and so increase the density of the collagen matrix. RAFT-stabilised collagen gels (RsC) were stored in PBS at 4°C for 24 hours, then measured and tested. At this stage, RsC gels were assumed to be incompressible materials[26,34]. For compressive DMA and rheology testing, 4 different batches of RsC gels (n=4), with three repetitions, were tested, each for five initial volume conditions, $[100; 150; 200; 250; 300]$ μl. For tensile DMA, 4 different batches of RsC gels (n=4), with three repetitions, were tested, each for four initial volume conditions, $[0.8; 1; 1.2; 1.5]$ ml. A total of 108 RsC gels were used. All gels were submerged in fluid during mechanical testing.

2.2 Gel Thickness Measurements

The height (thickness) of each RsC gel was measured using an optical contact angle meter (KSV CAM 200) to establish the new volume of the RsC gel after RAFT-stabilisation. Mean thickness for each RsC gel was determined from measuring three different positions within the gel.

2.3 Dynamic Mechanical Analysis (DMA) of the RsC gels

To quantify the viscoelastic behaviour of RsC gels, compressive and tensile Dynamic Mechanical Analysis (DMA) was performed at room temperature (21°C). The measurements were carried out using a BOSE-*ElectroForce*®3200 instrument equipped with a 250 g load cell and *Wintest*® DMA application software and frequency tests were performed from 1Hz up to 70 Hz.

Compressive DMA. The specimens were disk shaped RsC gels made in 96 well plates with a 6.4 mm diameter (Figure 2). To initiate an experiment, the upper plate was lowered until just touching the upper surface of the RsC gel sample, identified by the load cell properties. To investigate the linear viscoelastic response of the material, tests were run at a constant frequency of 5Hz for strain amplitudes of $[0.1 - 5]\%$ of the thickness of the RsC gel. To investigate the sweep frequency response, contact was established between the RsC gel and the load cell, the sample was precompressed by 15% of its thickness and then dynamically tested with sinusoidal compression over the following range of frequencies $[1 - 70]$ Hz for a displacement amplitude of $\pm 2\%$ of the thickness of the RsC gel.

Tensile DMA. For tensile experiments, the specimens were shaped using a cutter to provide a tapered shape with flared ends and a narrower central section (Figure 2). The flared ends of the gel were secured using titanium grips (Figure 2). At the start of the test, the specimens had a gauge length of 5.0 mm, a width of 4.0 mm, and a thickness dependent on the initial volume. To investigate the sweep frequency response, RsC samples were pre-extended by 10% of their gauge length and then dynamically tested with sinusoidal extension over the following range of frequencies $[1 - 70]$ Hz for a displacement amplitude of $\pm 2\%$ of the gauge length of the RsC gel.

2.4 Rheometry.

The rheology test was performed using a Discovery Hybrid Rheometer HR-3. An 8 mm diameter parallel plate configuration was used (Figure 2). The sweep frequency response was established at a frequency range $[1 - 20]$ Hz for a 2% applied shear strain to cylindrically shaped RsC gels (initial

volume =[150, 200, 250, 300] μl) and the complex shear modulus G^* was measured. The upper limit was determined to eliminate the effect of instrument inertia[35] leading to major variations.

To investigate linear viscoelasticity, the tests were run following the TA instruments protocol[36] at a constant frequency of 5Hz for an oscillation strain varying in the range of $[0.1 - 100]\%$.

Relation between Young's modulus E and Shear Modulus. The orientation of fibres within the stabilised collagen gel is random[30] so can be assimilated to homogeneous and isotropic material at a macro-scale. Also after stabilisation, gels were assumed to be incompressible, therefore, the Poisson's Ratio is $\mu = 0.5$.[34,37] These assumptions allow us to use the following relationship between the Young's modulus (E) and Shear modulus (G) in order to compare results from different types of testing:

$$E = 2G(1 + \mu). \tag{1}$$

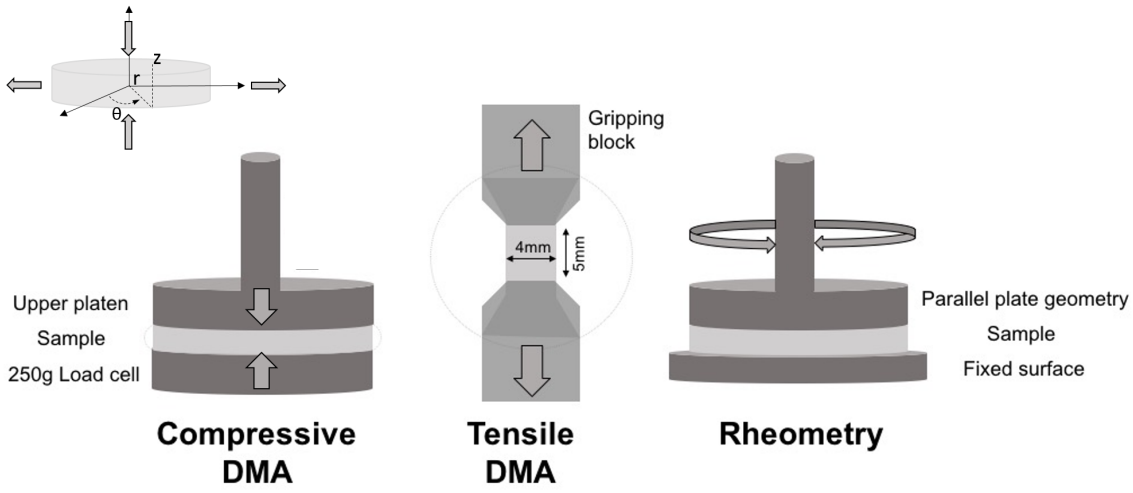


Figure 2: Schematic of the three different mechanical testing configurations run to investigate in detail the mechanical behavior of RsC gels in a cylindrical coordinate system (r,θ,z). Gels were submerged in fluid when tested.

2.5 Scanning Electron Microscopy (SEM) of the RsC gels

To determine the impact of mechanical testing on the previously reported[30] surface appearance and arrangement of collagen fibrils within the RsC gels, the samples were observed using SEM. Samples were fixed in 2% glutaraldehyde solution dissolved in 0.1M cocadylate buffer at $4 \circ C$ for

24 hours. The samples were dehydrated using a graded series of ethanol dilutions in water: 70% for 5 min; 90% for 10 min and 100% absolute ethanol for 3×5 minutes. Finally, the dehydrated RsC gels were mounted on specimen stubs, sputter-coated with gold/palladium alloy, and examined under SEM at 5 kV.

2.6 Statistical test

Results are expressed as mean ± standard deviation. A normality test was conducted. One-way ANOVA test was performed to evaluate the difference between means. A Mann-Whitney U test was used where data were non-normally distributed, as indicated in the relevant figure legend. Statistical significance was taken at $p < 0.05$

3 Results

3.1 Control of the stabilisation process

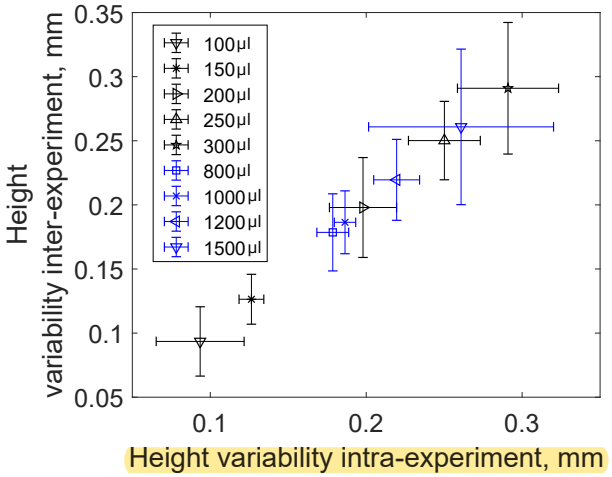
A range of initial volumes[13] were used in both 24 and 96 well plates, in order to establish the impact of initial volume on physical properties after the RAFT-stabilisation process (Table 1). To quantify the reproducibility of the protocol, the intra-experiment variability (SD_{intra}) for each initial volume (n=12), and the inter-experiment variability (SD_{inter}) describing the variability within all the different batches of gels of the same volume and shape (n=4) were tested. Figure 3 shows the SD_{intra} and SD_{inter} represented by the x-axis and y-axis standard deviation values respectively for the thickness of the RsC gels (3a), the percentage of fluid expelled after RAFT-stabilisation (3b) and the density of RsC gels (3c). Results are shown for gels with a top surface area of 210 mm^2 (RsC_{24}), and gels with a top surface area of 30 mm^2 (RsC_{96}).

Figure 3a shows the mean thickness (T) of stabilised gels for the nine different initial volumes. The height variability across all the samples was observed to be an average SD_{intra} value of $\pm 3.3 \times 10^{-2}$ mm for RsC_{24}, and $\pm 3.7 \times 10^{-2}$mm for RsC_{96}. Thickness variability across all the different batches was observed to be an average SD_{inter} value of $\pm 2.2 \times 10^{-2}$ mm.

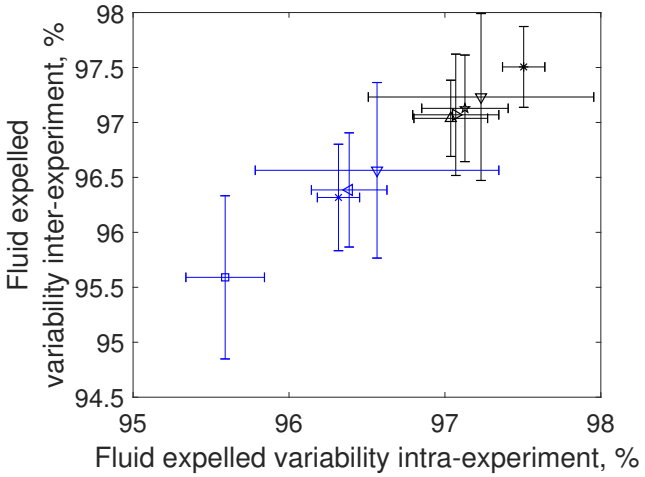
In addition, the fluid loss due to the stabilisation process was calculated via volume changes and is shown in Figure 3b. The stabilisation process used RAFT absorbers and produced dense RsC gels by expulsion of $97.2\% \pm 0.3$ fluid for RsC_{24} gels and $96.2\% \pm 0.3$ for RsC_{96}. The fluid expelled for the five initial volume conditions of RsC_{96}, and four initial volume conditions of RsC_{24}, were not significantly different. However, the amount of fluid expelled for RsC_{96} and RsC_{24} gels was significantly different ($p < 0.0001$).

Figure 3c shows the calculated collagen densities with the corresponding standard deviations. The average standard deviation across the RsC_{96} samples was ± 11.6 mg/ml and was ± 7.1 mg/ml for the RsC_{24} gels. The difference in collagen concentration for the fives initial volume conditions for RsC_{96} was not significant, and this was also true for the four initial volume conditions for RsC_{24}. However, the collagen densities for RsC_{96} and RsC_{24} gels were significantly different, with a p value < 0.0001.

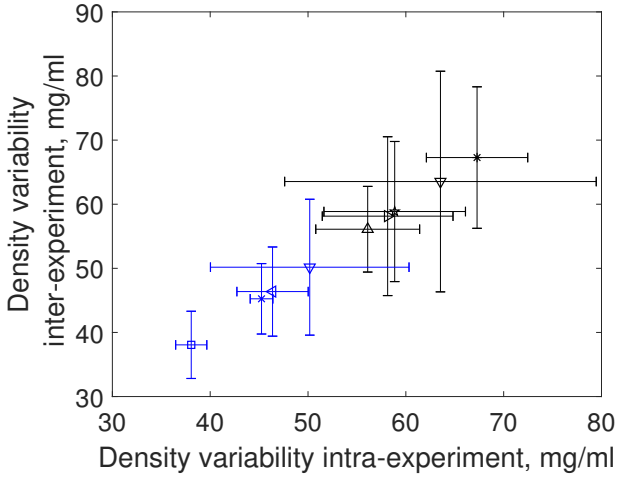
The protocol showed greater reproducibility for the samples with the biggest surface area with a standard deviation of only ±4.1 mg/ml, whereas the smaller gels had a standard deviation ±8.1 mg/ml. The gel density varied from the lowest value of 38.1 mg/ml for a stabilised thickness of $T_{Stabilised} = 0.25$ mm to the highest density value of 67.3 mg/ml for $T_{Stabilised} = 0.13$ mm (Table 1). Overall, the average density of the stabilised collagen gel was 61 mg/ml for RsC_{96} and 50 mg/ml for RsC_{24}. The initial gel geometry induced a significant difference in fluid removal and produced more diluted gels for RsC_{24} than RsC_{96} ($p < 0.0001$).



(a) Mean thickness of RsC gels in mm



(b) Fluid expelled after RAFT-stabilisation (%)



(c) Density of RsC gels in mg/ml

Figure 3: Physical properties of RsC gels for each initial volume (± SD), in blue for gels stabilised in 24 well-plates (RsC_{24}) and black for gels stabilised in 96 well-plates (RsC_{96}). A Mann-Whitney U test was used to analyse the variability inter-experiment.

	Top surface area (mm^2)	Initial Volume (ml)	$T_{Hydrated}$ (mm)	$T_{Stabilised}$ (mm)	Density (mg/ml)	Fluid Expelled (%)
RsC_{96}	29.76	0.10	3.38	0.09	63.53	97.23
		0.15	5.07	0.13	67.28	97.50
		0.20	6.76	0.20	58.14	97.07
		0.25	8.45	0.25	56.10	97.04
		0.30	10.14	0.29	58.86	97.13
RsC_{24}	201.00	0.80	3.98	0.18	38.06	95.60
		1.00	4.98	0.19	45.25	96.32
		1.20	5.97	0.22	46.37	96.38
		1.50	7.46	0.26	50.18	96.56

Table 1: Physical parameters defining the collagen matrix after RAFT-stabilisation for different initial volumes and in 24 (RsC_{24}) or 96 (RsC_{96}) well-plates.

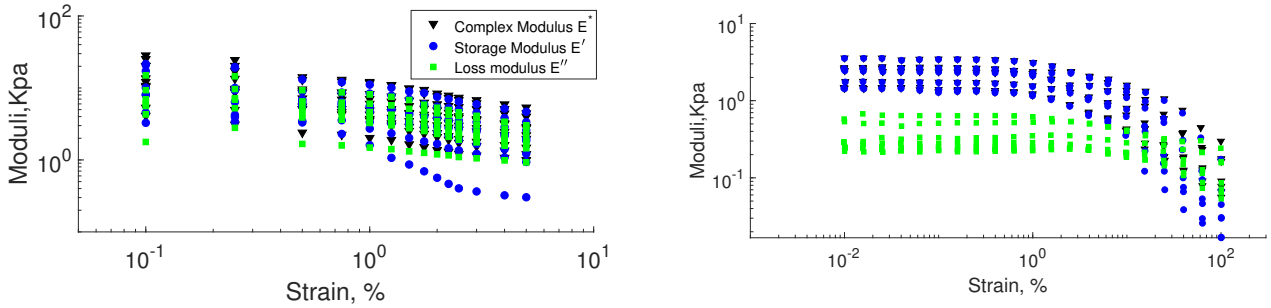
3.2 Mechanical properties of stabilised collagen gel

3.2.1 Linear viscoelasticity

The linear viscoelastic regime was explored using compressive DMA (Figure 4a) and rheology (Figure 4b) to determine suitable test conditions for subsequent experiments. For small strains, the compressive DMA results were subject to significant variability due to the limitations of the device in terms of noise. However, a linear elastic modulus trend was displayed from 0.01% up to 1.5% strain. So, the viscoelastic properties observed are independent of strain levels in this region, and beyond this point, the elastic modulus drops, and a constant viscosity coefficient can not be defined. Due to the variability of the results, the dynamic properties of the stabilised collagen gel were also analysed under an imposed shear stress. The linear relationship between strain and stress was sustained below approximately 2% strain for both compressive testing and rheology (Figure 4a and 4b). Therefore, the linear viscoelastic limit was chosen to be at 2% strain, above which the stress-strain relationship is non-linear.

3.2.2 Frequency sweep

Compressive DMA. The complex modulus values E^* (kPa) measured for the stabilised collagen gels under uniaxial compression are presented in Figure 5a for eleven different frequencies, in the range $[1 - 70]$ Hz. The data shown are the mean numbers of $n = 4$ different batches of gels with three repetitions for each of the five different initial volume conditions. A continuous increase of E^* with increasing frequencies was observed with no significant differences between initial volumes. For each frequency, E^* ranges were overlapping. This indicates that all RsC gels



(a) Strain sweep, compressive DMA testing

(b) Strain sweep, rheometry testing

Figure 4: Determination of the linear viscoelastic region of RsC gels at 21°C and constant frequency of 5 Hz (a) for an oscillation strain varying in the range $[0.1 - 5]\%$ for compressive DMA testing (b) or an oscillation strain varying in the range $[0.1 - 100]\%$ for rheometry testing.

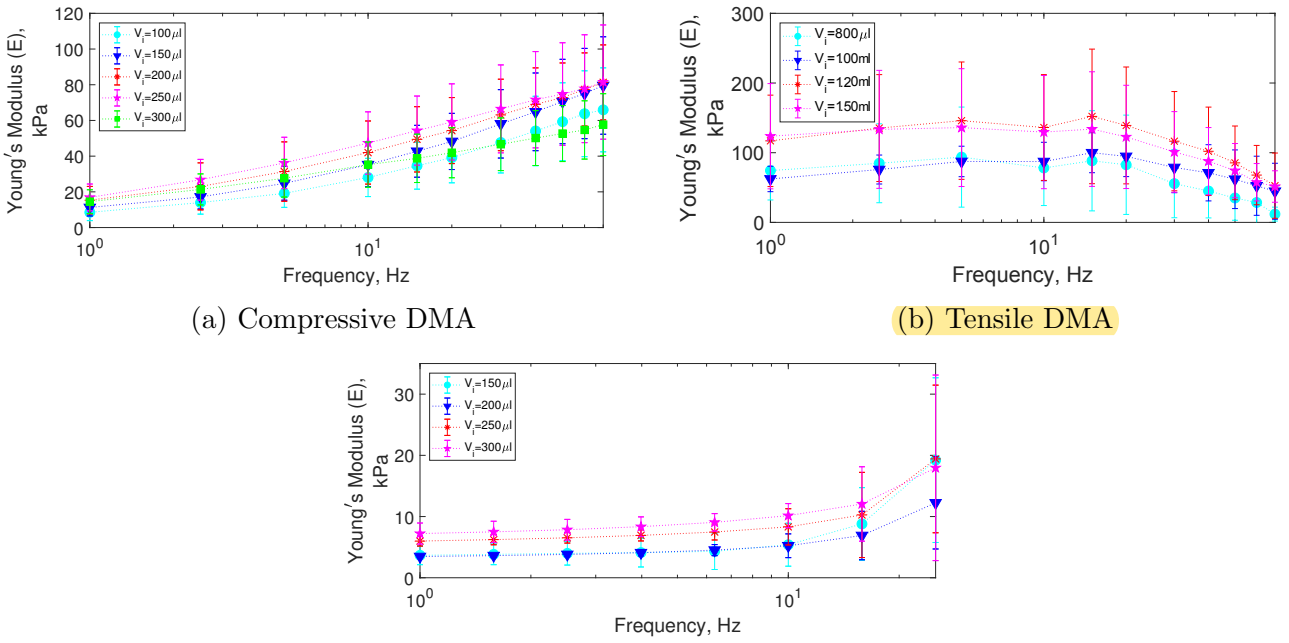
behaved similarly under compression.

Tensile DMA. Figure 5b shows the effect of a frequency sweep ($[1 - 70]$ Hz) on E^* under tensile testing for each of the four different initial volume conditions. E^* remained roughly constant (100 kPa) up to the yield point at 15 Hz before collapsing, characterising permanent elongation. All RsC gels exhibited a similar behaviour under tensile DMA.

Rheometry. Four different initial volumes were analysed using rheometry (Figure 5c). The complex shear modulus G^* values obtained were converted to E^* using Equation (1). As observed under tensile testing, E^* remained constant up to 15 Hz across all samples (Figure 5c).

RAFT-stabilised gel behaviors in each of the three dimensions were analyzed using different techniques. Under uniaxial compression (Figure 6a), the RsC gels had a viscoelastic response. The viscous component of the loss modulus (E") ranged from 10 to 40 kPa, which was about 2.5 kPa higher than the elastic component of the storage modulus (E'), which was in the range 1 to 30 Hz. Comparatively, from 40 to 70 Hz, the behavior became more viscous than elastic.

By comparison, Figure 6b shows the mechanical behavior under tensile testing. The elastic component, E', clearly predominates as the value of the loss modulus was almost zero for all the frequencies. So under tensile load, the dense collagen matrix gels display elastic behavior. Also,



(a) Compressive DMA

(b) Tensile DMA

(c) Rheology

Figure 5: The Young's Modulus (E, kPa) as a function of the frequency (Hz) for each initial volume condition of the RAFT-stabilised collagen gels (a) for a sinusoidal compression [1-70]Hz (b) for a sinusoidal extension [1-70]Hz (c) for an oscillating shear strain [1-15]Hz.

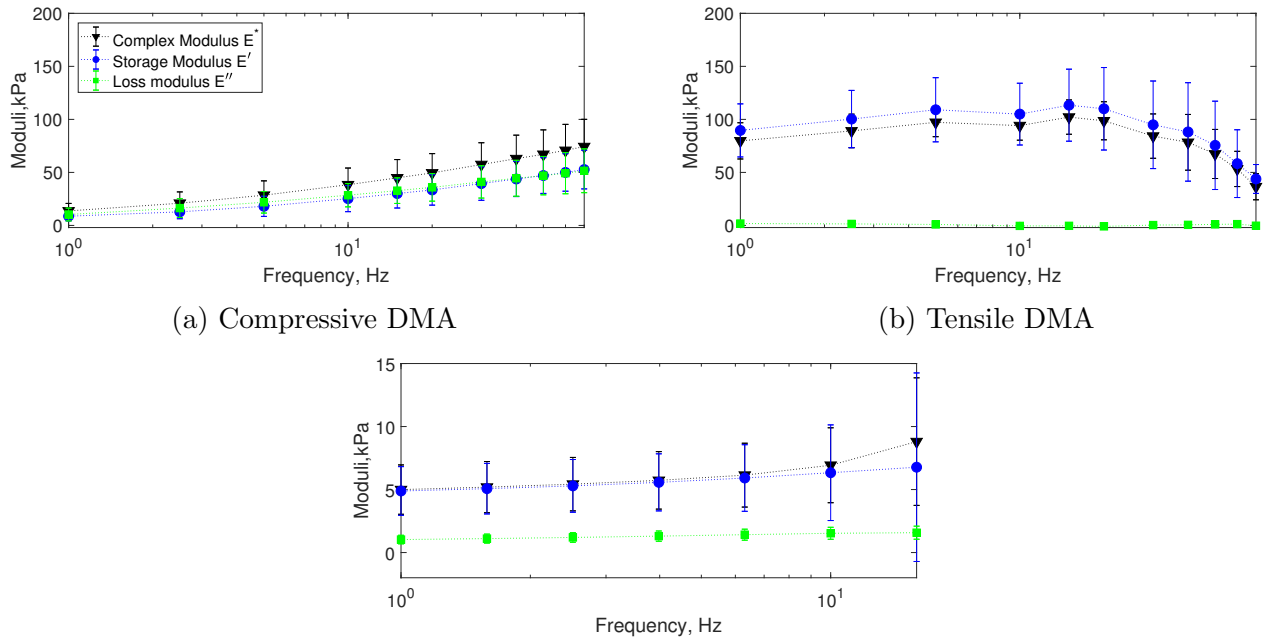
we can report that the tensile modulus is 1.4 to 7.7 times bigger than the compressive modulus.

For the rheometry measurements (Figure 6c), the behavior was viscoelastic as the E" value was non negligible. The stiffness of the gel under shear stress was around 5 kPa, so 2.6 times softer than under compression, for the corresponding range of frequency ($[1-15]$ Hz).

3.2.3 Effect of frequency on mechanical properties

In order to characterise the relationship between mechanical properties and frequency, a regression analysis was performed to find an empirical expression which closely correlated the experimentally measured data to the strain rate. A set of formulae was produced to describe the correlation between the Young's modulus (E) and the frequency for rheometry, compressive and tensile DMA, through numerical fitting. The effects of the frequency sweep on the Young's modulus for the compressive DMA, tensile DMA and rheology were formulated as :

$$E_{compressive}(f) = 15.93 * \log(f) + 4.348, \quad R^2 = 0.9803, \tag{2}$$



(a) Compressive DMA

(b) Tensile DMA

(c) Rheology

Figure 6: Complex Modulus (E*, kPa), storage modulus (E′, kPa) and loss modulus (E″, kPa) as a function of frequency (Hz) at strain amplitude of ±2% for (a) Compressive DMA (b) Tensile DMA and (c) Rheology testing.

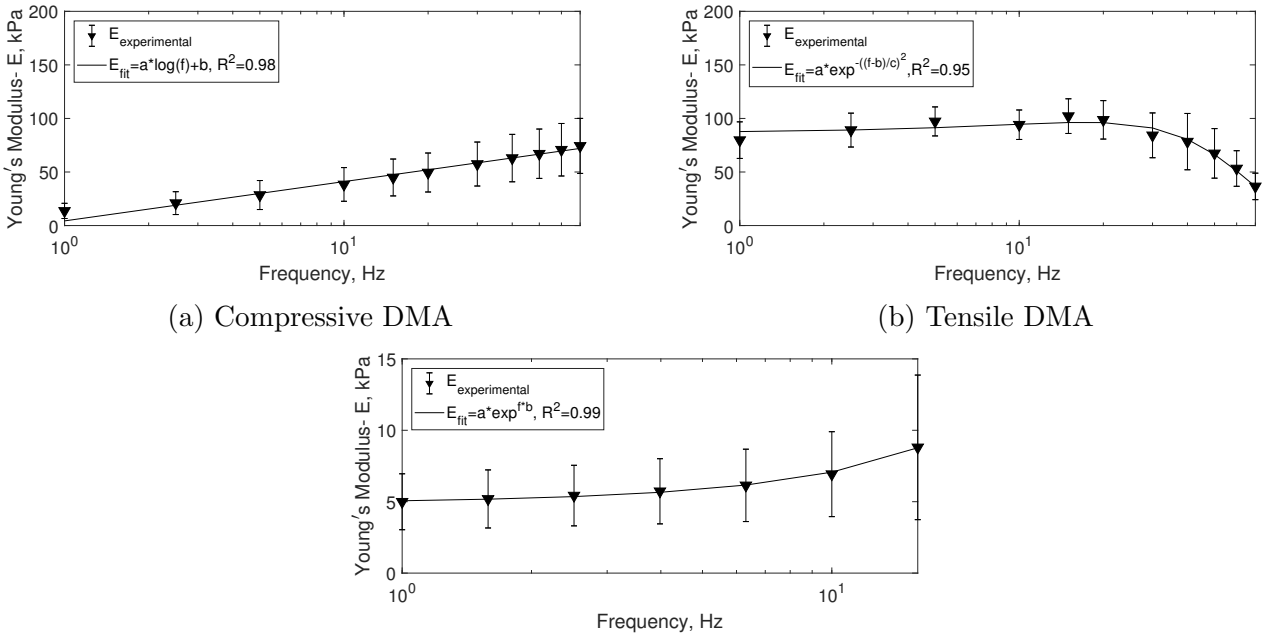
$$E_{tensile}(f) = 96.37 * \exp^{-((f-17.36)/53.41)^2}, \quad R^2 = 0.9525, \tag{3}$$

$$E_{rheology}(f) = 4.881 * \exp^{(f*0.037)}, \quad R^2 = 0.9966, \tag{4}$$

where the Young's Modulus E is in kPa and the frequency f is in Hz (Figure 7a-7c). The fitted logarithmic, Gaussian and exponential curves are appropriate models to describe the effect of frequency sweep on the stiffness of RsC gels, and the comparison with the experimental data is shown in Figure 7.

3.2.4 Morphological characterisation

Figure 8 shows SEM micrographs of the top surface of RAFT-stabilised collagen gels after various types of mechanical loads. Figure 8a is the surface image of a RsC gel and Figures 8b,8c and 8d show RsC gels after compressive, tensile and rheology analysis respectively. Before any testing, the RsC gels displayed a randomly orientated porous architecture with visible and entangled fibrils. The same pattern was observed for the gels after tensile testing. RsC gels after rheology and



(a) Compressive DMA

(b) Tensile DMA

(c) Rheology

Figure 7: Fitted curves to describe the relationship between Young's modulus (E, kPa) and frequency for (a) Compressive DMA (b) Tensile DMA and (c) Rheology.

compressive testing (Figure 8b and 8d) demonstrated a more compact and less porous surface matrix where the collagen fibrils seemed to have merged together and were harder to distinguish. The images show that the seemingly random organisation of the fibrils on the surface was not disturbed by the mechanical characterisation analysis.

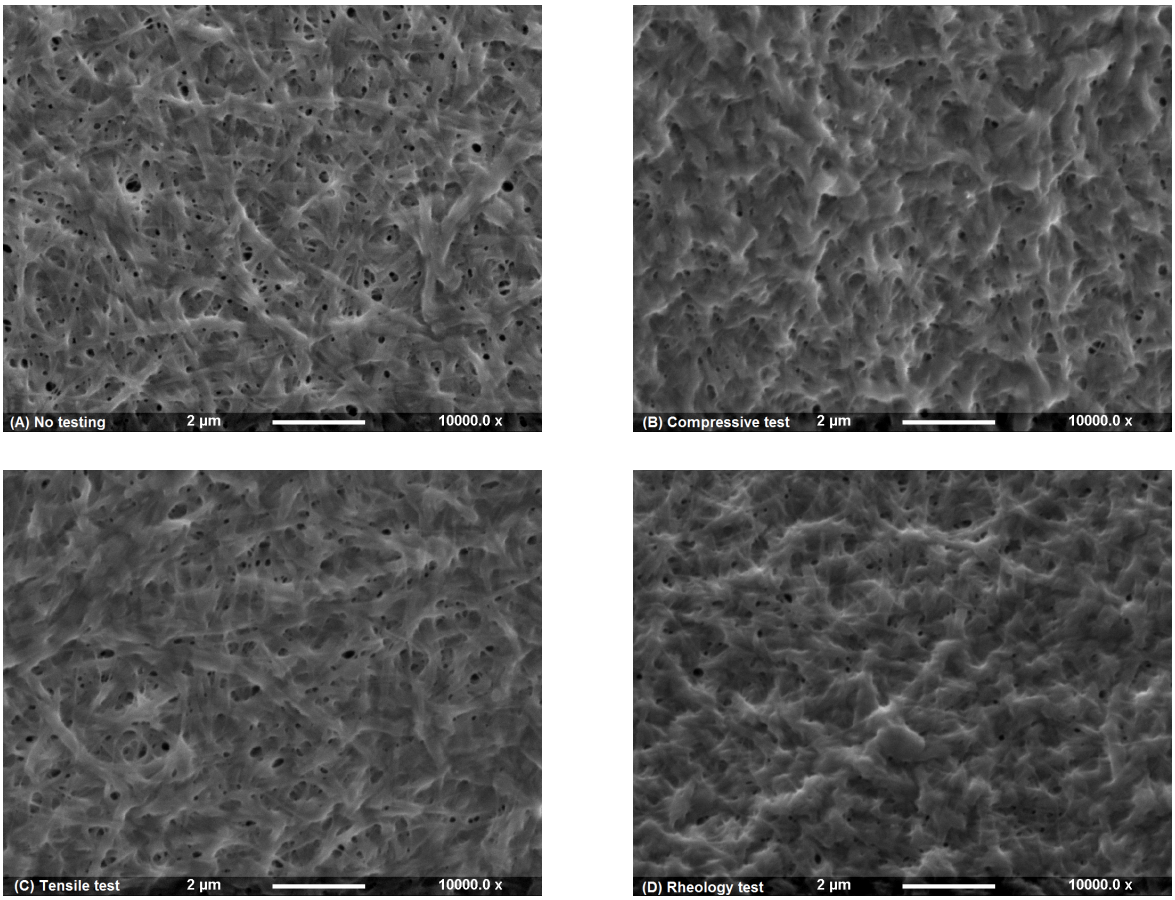


Figure 8: Scanning electron microscopy images. Top surface view of RAFT-stabilised collagen (RsC) gels (A) Before testing then following (B) Compressive load, (C) Tensile load, and (D) Rheology. (Scale bar = 2 μm).

4 Discussion

The RAFT-stabilisation process[26,31] was applied to mechanically weak, fully-hydrated collagen hydrogels[15,26], as this has been shown previously to produce collagen structures with improved mechanical stiffness comparable to soft tissue values, by the rapid removal of fluid.[10,26,30] This technique has many tissue engineering applications[31] and is now standardised, rapid, simple and not experience-dependent, through the use of commercially available RAFT-absorbers. The stiffness parameters characterising this type of stabilised collagen within the literature[30,38,39] have been defined using multiple methods, across a range of gel compositions, and therefore values are not directly comparable.

Levis et al[31] provided a qualitative analysis that showed the dense collagen matrices obtained after RAFT-stabilisation were able to withstand handling and be attached to *in-vivo* like tissues using fibrin glue or similar. In this study, we have assessed for the first time the physical and mechanical properties of RAFT-stabilised collagen gels using a comprehensive range of quantitative measurements techniques. Physical properties, such the collagen density and the fluid loss due to the stabilisation, were assessed for two different dimensions of gels made in standard multi-well plates (RsC_{24} = 29.76 mm^2 and RsC_{24} = 201 mm^2) with a range of different initial volumes of collagen solution.

It has been reported previously that during plastic compression about 97% of fluid is expelled[30] by a downward fluid flow[26], which is consistent with the 96-97% measured in this study for the upward fluid expulsion. Furthermore, for RsC_{96} gels, 1% more fluid was removed compared to the RsC_{24} gels, and this has a direct impact on the final concentration of collagen gel (61 $mg.mL^{-1}$ for gels in RsC_{96} versus 50 $mg.mL^{-1}$ for gels in RsC_{24}). This phenomenon could be due to the use of different sizes of hydrophilic porous absorbers[31] for stabilisation. The surface area covered for the RsC_{24} gels is larger and so the weight of the fluid absorbed is differently distributed. For a given surface area and volume, the thickness of the RsC gels can be consistently predicted and so defined using industrial absorbers.

The dense collagen type I structure retains approximately 3% of the initial fluid volume trapped within the fibres, and displays a viscoelastic behavior under compressive and shear stress. The

RsC gels were two orders of magnitude stiffer than fully hydrated 1.5 mg.mL^{-1} collagen gels under a 2% shear stress reported in previous studies.[15] This result confirms the production of a stiffer matrix by the fluid removal process[26,30] compared to weak fully-hydrated collagen gel.[26,31] The mechanical characterisation of the RsC gels under compressive and shear dynamic testing revealed material properties similar to those of various soft tissues, e.g. lung (2.5-9 kPa), cornea (50 kPa)[28,40–42], making these gels attractive for various medical applications.

Under tensile stress, the RsC gels displayed a purely elastic behavior at frequencies under 15 Hz and were permanently deformed at higher frequencies. Consistent with the Buffinton et al[32] study, the yield point is hypothesised to be reached due to decreasing hydration level of the gels, which causes a strength loss inducing permanent deformation that potentially leads to a material fracture. The collagen structure obtained is also 20 times stronger (approximately 100 kPa) than fully-hydrated collagen gels (5 kPa for a collagen concentration of 1.5 mg.mL^{-1} [20]) under tensile testing (at 5 Hz and 2% strain), with is consistent with other studies[30,32] reporting increased tensile strength after stabilisation.

The results reported here are consistent with previous studies showing the moduli of hydrogels measured in tension is higher than moduli measured in compression[32] which can be explained by the flow of fluid within the hydrogel during the testing. For RsC gels, the tensile modulus was measured to be 1.4 to 7.7 times stiffer than the compressive modulus. This confirms that RsC gels have potential to be taken forward for tissue engineering approaches.

The data reported here can be used to predict the behavior of RAFT-stabilised collagen gels under different mechanical loads, for example to identify limitations such as material failure during tissue engineered scaffold applications.[43] The numerical fitting performed on the experimental data provides a set of predictive formulae (Equation 2 to 4) to describe the correlation between the Young's modulus, E, and the frequency for rat tail collagen. This will be of use in the field of tissue mechanics, as well as studies establishing the fundamentals of the cell-substrate interaction using collagen-based materials.

SEM images indicated random fibrils orientation so the RsC matrix can be assimilated to an isotropic material at a macro scale. The fibrils have a less distinct fibrillar structure to that shown

in the literature for the plastic compression protocol.[30] In Neel et al[30], a dense and compacted appearance of fibrils was observed after double compression. A similar organisation was observed for the RsC gels after both compressive DMA and rheology. The recurrence of these tests for each frequency can cause a reorganisation of the collagen fibrils and leads to analogous pattern that resembles gels after a second compression.[30] Also, the extension test on the RsC gel did not impact the random orientation of the collagen at the fibrils scale[30].

5 Conclusion

It has been demonstrated that stabilisation of collagen hydrogels using RAFT absorbers produces reproducible dense material with similar physical characteristics to those reported previously using other methods of plastic compression. The initial plate geometry affects the final collagen matrix properties (fluid loss and collagen density). A comprehensive set of mechanical tests have shown that this material exhibits different tensile, compressive and rheological properties and that varying the initial volume of collagen solution does not significantly impact these properties for a given geometry. It exhibits viscoelastic behavior under compressive and shear stress and a predominantly elastic behavior under tensile stress. In addition, the data suggest that irreversible structural changes may occur at frequencies higher than 15 Hz. Moreover, the experimental data obtained for each mechanical test have been correlated with the frequency sweep through the identification of an empirical relationship. These results can be used for numerous applications in the area of tissue engineering, for example to correlate cell behavior with matrix properties and to understand the fundamentals of material-tissues interactions in regenerative medicine.

6 Disclosure section

The authors declare no conflict of interest.

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