MULTISCALE CHARACTERIZATION OF RELAXATION TIMES OF TISSUE SURROGATE GELS AND SOFT TISSUES

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ABSTRACT

The development of tissue surrogate materials that can replicate the mechanical response of biological soft tissues is of increasing interest. Such materials are required to enable robust experimental capabilities for evaluating next-generation armor systems that are critical to the survivability of the Soldier with regard to mechanical impact forces, including small caliber projectiles, fragments, and blast waves. Here, we characterize the viscoelastic stress relaxation response of heart tissue and candidate tissue surrogate gels, thermoplastic styrenic triblock/diblock copolymers. We explore the effects of gel composition and probe contact area on the relaxation modulus *E(t)* and characteristic relaxation times τ of these tissue surrogate gels, with comparisons to tissue response. We observe that *E(t)* varies only as a function of vol-% triblock, whereas τ varies as a function of volume of the material probed. The latter variation is explained by the coupling between viscoelastic material deformation and probe-material adhesion. Finally, we find that heart tissue is more compliant and relaxes more quickly than the candidate tissue surrogate gels studied here.

1. INTRODUCTION

To design armor with superior protection, it is necessary to understand the response of soft tissues under impact forces. For over 40 years, ballistic gelatin (BG) has been used as soft tissue simulant to evaluate effect of firearms on soft tissue (Nicholas and Welsch, 2004). However, due to the unstable mechanical properties of BG as a function of time and temperature (MacPherson, 1994), researchers at the US Army Research Laboratory (US-ARL) are developing new tissue surrogate gels (Juliano et al., 2006; Moy et al., 2006). These thermoplastic styrenic gels comprise ABA triblock and AB diblock copolymers that can form a micellar structure connected with physical crosslinks by using a block selective solvent (Nguyen-Misra and Mattice, 1996), which was mineral oil in those studies. Advantages of such physically associating gels are the ability to tailor their structure to mimic the viscoelastic properties of various soft tissues, and the increased mechanical stability in air that derives from the nonvolatile nature of the solvent as compared to hydrogels.

Previous mechanical studies on styrenic thermoplastic gels have included macroscale and microscale quasistatic indentation experiments, microscale dynamic tests (Juliano et al., 2006), macroscale high rate impact tests via split Hopkinson pressure bar (Moy et al., 2006) and macroscale stress relaxation tests (Hotta et al., 2002; Mandare et al., 2005; Quintana et al., 2002). Previously, we have conducted high rate dynamic impact indentation tests on both soft tissues and a new system of styrenic triblock/diblock gels comprising styrene (S) and either ethylene/butylene (EB) or ethylene/propylene (EP), to compare penetration resistance and energy dissipation capacity of these materials (Kalcioglu et al., 2010). We found that by changing the composition of these gels, the energy dissipation capacities of fully hydrated soft tissues could be matched by these gels.

To aid the design of this new system of potential tissue surrogate gels, in this study we focus on time dependent mechanical properties at lower deformation rates. Specifically, we report multiscale stress relaxation experiments on thermoplastic gels comprising SEBS triblocks and SEP diblocks of varied vol-%, and on murine heart tissue. Our aim is to study the effect of vol-% triblock and probe contact areas on relaxation modulus *E(t)* and characteristic time scales τ. To explore the effect of contact area, we compare the responses measured via macroscale rheometry, mesoscale instrumented indentation, and microscale atomic force microscopy (AFM)-enabled indentation. Additionally, we utilize deposition of noninteracting fluids on the sample surface to reduce the effects of adhesive forces, which are more dominant at the smaller scales of probe-surface interactions. The procedures outlined in this study will

allow further multi-scale study of relaxation behavior of the tissue surrogate gels and soft tissues, with the ultimate goal of developing gels that can mimic key viscoelastic properties of soft tissues.

2. EXPERIMENTAL METHOD & ANALYSIS

2.1 Materials

Potential tissue surrogate gels are formed from styrenic block copolymers (Kraton Polymers) comprising styrene (S) and either ethylene/butylene (EB) or ethylene/propylene (EP). Copolymer-based gel samples were made by mixing SEBS triblock copolymer (Kraton G1652) with SEP diblock copolymer (Kraton G1701). The copolymers were dissolved at elevated temperature in light mineral oil (Mallinckrodt Chemicals, St. Louis, MO) at a volume ratio of 20:80 polymer to oil, forming gels upon cooling to room temperature. Each polymer-oil mixture was placed in a vacuum oven at 150°C and fully dissolved over 6 h with stirring every hour. The melt was then poured onto a flat surface to cool and gel. Light mineral oil is a block selective solvent, i.e., a good solvent for the rubbery blocks and a poor solvent for the styrene blocks, and has a low vapor pressure facilitating development of physically associating gels that are stable in ambient conditions. Samples were mixed at triblock:diblock volume ratios of 100/0, 75/25 and 50/50. Heart tissue was harvested from healthy male, adult Sprague-Dawley rats (250-350 g). All experiments involving animals were approved by the university IACUC protocol and compliant with NIH guidelines for animal care.

Heart tissue exhibits a layered or striated structure that is heterogeneous at the microscale (Ashley and Niebauer, 2005). The synthetic gels studied here exhibit structural heterogeneity only at the nanoscale, in terms of micelle formation (Fig. 1a) but are structurally homogeneous at the macroscale (Fig. 1a inset). As schematized in Fig. 1b, AB (SEP) diblock chains contain the A block, which form the micellar core, and the B block, which extends away from the micelle into the solution (i.e., adding no network connectivity). In contrast, the B block of the ABA (SEBS) triblock chains can form either bridges, when connecting A blocks that are in neighbouring micelles; loops, when both A blocks are in the same micelle; or chains, when only one A block is in a micelle with the rest of the triblock extending into solution (Quintana et al., 2002). Therefore by tuning the relative concentration of triblock to diblock, the effective crosslinking density within these synthetic gels can be manipulated.

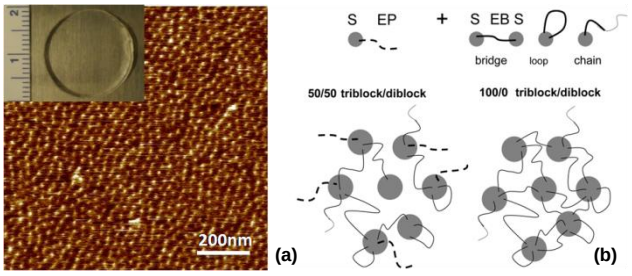

Fig. 1 (a) Atomic force microscopy phase image of 100/0 triblock/diblock gel showing segregated micellar domains. Inset: macroscale photograph of gel disc of diameter ~2 cm appears homogeneous. (b) Schematic configuration among ABA/AB triblock/diblocks in gel as a function of vol-% triblock. (Adapted from Kalcioglu et. al., 2010).

2.2 Experimental:

Structural and mechanical characterization of the gels was carried out in ambient air, as required for use in intended tissue surrogate applications. All tissues were stored in Krebs-Hensleit buffer immediately after excision and throughout all experiments reported herein. All experiments were conducted within 3 hours post mortem at 37^{o}C.

Macroscale stress relaxation testing was conducted using a rheometer (AR 2000, TA Instruments) for the gels and a universal load frame (Instron) for the tissues. Gel samples were punched (15 mm diameter) from ~4 mm thick sheets. A thin layer of silicone oil (1000 cst) was used to lubricate sample/plate contact regions. For heart tissue, discs of 8 mm diameter and thickness of 3 to 4 mm were prepared using a surgical punch. For the gels, applied strains were 7%, and for heart tissue, strains were held at 10% in order to confine the material behavior to the linear viscoelastic regime.

Mesoscale load relaxation testing was conducted using an instrumented indenter (TriboIndenter, Hysitron, Inc.) with a truncated cone of radius R = 50 µm. Indentation depths h were held constant at 500 nm with a rise time of 1 s and a hold time of 100 s. Because the change in the contact areas was 1% over this hold period, the probe geometry was approximated as a flat punch. Applied strains were calculated as the ratio h/R and were ~1%.

Microscale AFM-enabled indentation and load relaxation experiments were performed only on the 100/0 triblock/diblock gel (3D-MFP, Asylum Research). Modified plateau probes (PL2-FM, Nanosensors), which have nominal spring constants of ~1-5 N/m, were used. PL2-FM probes have geometries similar to a "flat punch" with a plateau diameter of 1.8 µm ± 0.5 µm. Experiments were conducted in air, in water, and in PBS+0.2%

Pluronic F108 solution. Pluronic® F108 is a nonionic polyethylene-polyoxyproplyene (PEO-PPO) block copolymer surfactant terminating in primary hydroxyl groups. Indentation experiments were conducted with a maximum force of 180 nN and a constant piezo velocity of 2.6 μm/s with no dwell period. For load relaxation experiments, indentation depths were held constant at 1 μm with a rise time of 1 s and a dwell period of 60 s to minimize drift. AFM phase images were acquired in air using AC mode at a scan rate of 0.5 Hz with sharp silicon cantilevers (OMCL AC240TS-W2, Olympus). Images of the gel-probe system were also acquired in air via optical microscopy.

2.3 Analysis

For macroscale stress relaxation experiments, relaxation modulus *E(t)* can be calculated as:

$$E(t) = \sigma(t)/\varepsilon_o \quad (1)$$

where σ is instantaneous stress calculated from force normalized by cross-sectional sample area and ε_o is applied strain. For mesoscale and microscale load relaxation experiments, a linear viscoelastic model for indentation of a rigid flat punch into a homogeneous, isotropic material was used to fit the load vs. time response to calculate *E(t)* which was expressed as a general Maxwell model (Du et al., 2010). To calculate the relaxation time scales τ, normalized stress (at the macroscale) or normalized force (at the mesoscale and microscale) vs. time responses were described reasonably well using a stretched exponential of the KWW type (Williams and Watts, 1970):

$$x(t) = x_o e^{-(t/\tau)^\beta} \quad (2)$$

where β describes the distribution of relaxation times with values between zero and unity.

For microscale AFM-enabled indentation experiments, Young's elastic modulus *E* was calculated by fitting the loading part of the load vs. time response, using the solution for indentation of a rigid flat punch indenter into a homogeneous, linear elastic and isotropic material (Harding and Sneddon, 1945):

$$E = P(1-\nu^2)/(2hR) \quad (3)$$

where *P* is force and *ν* is Poisson's ratio that we assumed to be valued 0.5 for these gels.

3. RESULTS

3.1 Macroscale Stress Relaxation

Macroscale relaxation modulus calculated via Eq. 1 varied significantly as a function of composition (Fig. 2a). First, instantaneous modulus E_o calculated from *E(t=0)* increased as the triblock vol-% was increased. This stiffening can be explained via the difference among samples in terms of the number of bridges formed between micelles that act as physical crosslinks (elastic springs). Only triblocks are capable of forming bridges, and thus increasing the triblock concentration increases the crosslink percentage as seen in Fig 1b. Second, heart tissue exhibited the most compliant behavior. In order to match tissue response, theoretically these gels can be rendered more compliant by increasing vol-%diblock. However, in this material system, complete gelation could not be achieved for triblock composition <50 vol-%.

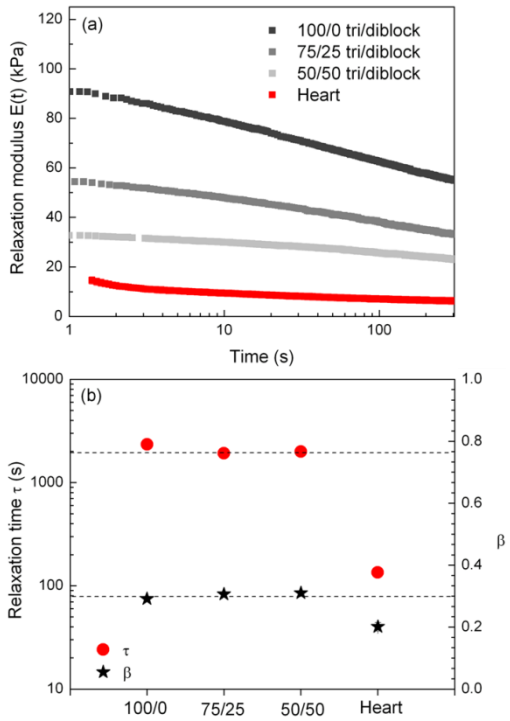

Fig. 2 (a) Macroscale relaxation modulus of tissue surrogate gels and heart tissue. (b) Calculated τ and β by fitting stress vs. time response via Eq. 2.

Fits of the macroscale stress vs. time response via Eq. 2 showed that relaxation times τ were on the order of 1000 s (Fig. 2b). The long relaxation times found here is consistent with macroscale stress relaxation experiments conducted on other styrenic triblock gels (Hotta et al., 2002). Both τ and the distribution of relaxation times β were not strongly dependent on vol-% triblock. The relatively low value of β (compared to unity) indicated that there are multiple relaxation processes

activated in this system. Further, heart tissue exhibited a much faster relaxation and a wider distribution of relaxation times.

3.2 Mesoscale Load Relaxation

Mesoscale relaxation moduli of the triblock/diblock gels confirmed this macroscale result: increasing the vol-% triblock stiffens the gel (Fig. 3a). Although the contact areas were orders of magnitude smaller than in those macroscale experiments (100s of μm^2 vs. mm^2 for the latter case), for a given sample, the difference in macroscale and mesoscale $E(t)$ was less than 50%. This general agreement between these lengthscales was expected in that, in both cases, contact areas were much larger than the micelle dimensions. Therefore, at these lengthscales, the gels exhibit a homogeneous mechanical response.

As in the macroscale experiment, the calculated mesoscale relaxtation times were also on the order of 1000 s, and did not exhibit a significant dependence on vol-%triblock (Fig. 3b). The distribution of relaxation times τ was on the order of 0.2-0.3, also similar to macroscale results.

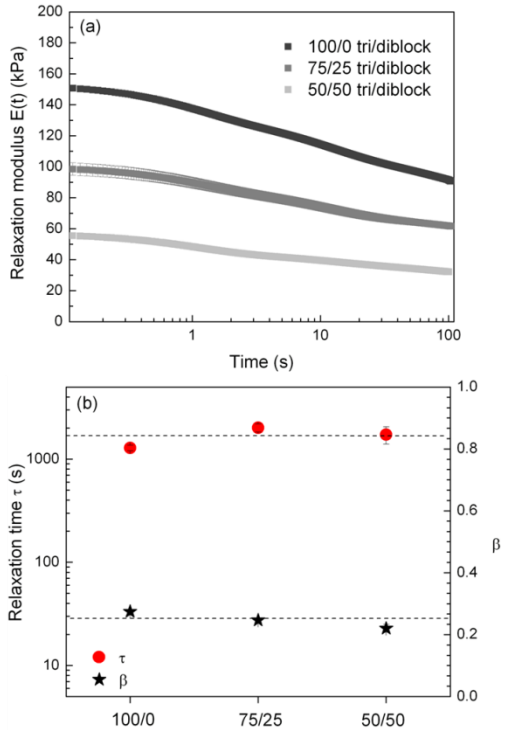

Fig. 3 (a) Mesoscale relaxation modulus of triblock/diblock gels as a function of vol-%triblock. (b) Calculated τ and β as a function of vol-%triblock by fitting load vs. time response via Eq. 2. Data represented as mean ± standard error.

3.3 Microscale AFM-Enabled Load Relaxation

AFM-enabled relaxation experiments were conducted on the 100/0 triblock/diblock gel to investigate the effects of lengthscale on the characteristic relaxation time scales. Note that at these small contact areas, other factors such as adhesive forces between the probe and the sample can play a role in the measured relaxation times (Fig. 4a inset). To investigate this potential contribution, experiments were conducted in air, in water, and in PBS+0.2% Pluronic® F108. In general, pluronic surfactants adsorb to hydrophobic surfaces with the PEO block extending into the solution and the more hydrophobic PPO block anchoring as loops and trains (Wang et al., 2002). Both in air and in water, adhesive forces between the gel and the probe were comparable to the applied maximum loads (Fig. 4a). In PBS+0.2% Pluronic® F108, these adhesive forces were negligible when compared to maximum applied loads.

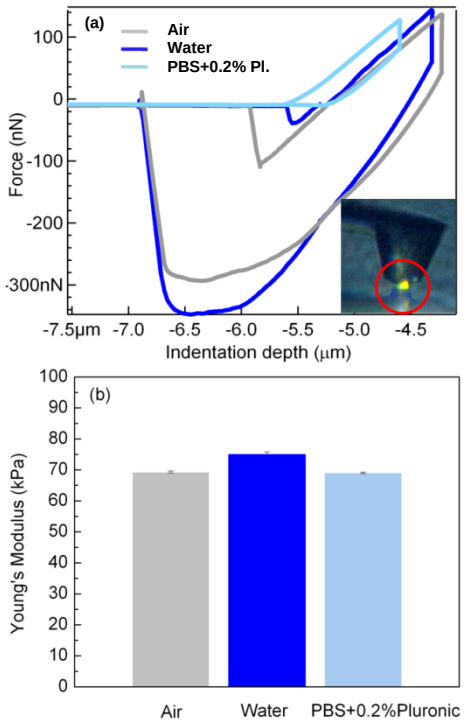

Fig. 4 (a) Representative load vs. depth responses of 100/0 triblock/diblock gel as a function of immersion environment. Inset: optical microscope image of the gel adhering to the probe during AFM experiments in air. (b) Young's elastic modulus of 100/0 triblock/diblock gel as a function of immersion environment. Data represented as mean ± standard error.

To investigate the effect of immersion environment on the surface mechanical properties of the gel, quasistatic indentation experiments were conducted via AFM. Young's moduli E were calculated from the load vs. indentation depth responses via Eq. 3. The results

showed that *E* did not change significantly as a function of environment (Fig. 4b). Thus, we concluded that this surfactant reduced adhesion without changing the surface mechanical properties significantly.

Stress relaxation experiments were conducted in all three environments. The raw load vs. time responses showed that maximum forces obtained for all cases was comparable (Fig. 5a). It was observed that both in air and in water, environments conducive to higher adhesive forces, force relaxed more quickly. On the other hand, in PBS+0.2% Pluronic® F108 which enabled negligible adhesive forces, force relaxed more slowly.

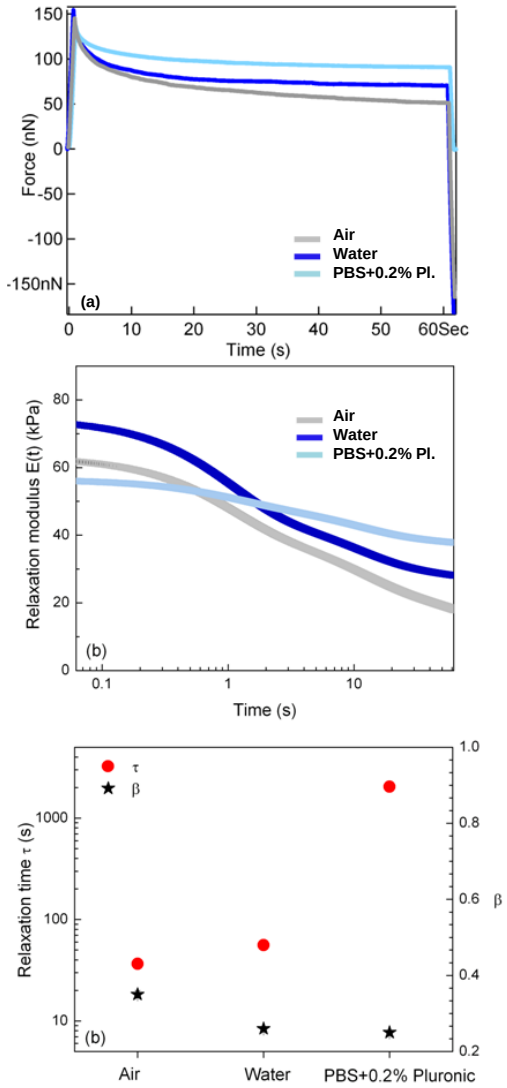

Fig. 5 (a) Representative load vs. time responses of the 100/0 triblock gel in air, in water, and in PBS+0.2% Pluronic® F108. (b) Microscale relaxation modulus of 100/0 triblock/diblock gel as a function of immersion environment. (b) Calculated τ and β in different immersion environments.

Quantitative analysis of *E(t)* further demonstrated that immersing the sample in these solutions does not affect the instantaneous elastic moduli appreciably: E_o in air (63 ± 0.7 kPa) and in PBS+0.2% Pluronic® F108 (56 ±0.4 kPa) were comparable (Fig. 5b). In contrast, relaxation moduli *E(t)* of this gel exhibited similar behavior in air and in water, but changed much more slowly over time in Pluronic® because the forces relaxed more slowly in the absence of appreciable probe-gel adhesion.

The relaxation times calculated from load vs. time responses showed that both in air and in water, τ was on the order of 10s of seconds (Fig. 5c). This relaxation time is two orders of magnitude smaller than that determined from macroscale and mesoscale experiments. In PBS+0.2% Pluronic® F108, τ was calculated to be on the order of 1000 s, and was thus comparable to that measured in larger scale experiments.

4. DISCUSSION

4.1 Effect of Composition on *E(t)* and τ

Both macroscale and mesoscale analyses showed that the relaxation modulus increases with increasing vol-% triblock. This result is explained by the replacement of SEBS chains by SEP chains, which induces a decrease in the number of bridges that form a network connecting the physical crosslinks (micelles). In contrast, τ and β did not show dependence on the vol-% triblock, i.e., on the stiffness of the gels. Similar results have also been shown previously on biological cells: τ and β calculated from stress relaxation experiments via AFM on human hepatoma cell line HEPG2 were independent of the estimated cell elastic modulus *E* (Okajima et al., 2007). In general, relaxation time is strongly dependent on factors that affect molecular mobility within the material and is correlated inversely to the rate of molecular motion. For triblock systems, τ depends on temperature, endblock length, polymer volume fraction and aggregation number of micelles (number of endblocks per micelle) (Seitz et al., 2007). For the systems studied here, we did not vary the temperature, endblock length, or polymer volume fraction. Further, addition of diblock into a triblock system is expected to decrease the number of bridges and thus decrease the elastic modulus; however, it is not expected to affect the micelle aggregation number (Seitz et al., 2007). This lack of change in the micelle structure explains why τ and β did not depend on the triblock vol-%. Heart tissue exhibited more compliant behavior and shorter relaxation times than all the gels considered here. Although further increases in gel compliance cannot be achieved by changing the diblock ratio due to incomplete gelation, other factors such as chain length and polymer volume fraction can be tuned for designing tissue

surrogate gels with stiffness and relaxation time constants that are optimum for the application of interest.

4.2 Comparing *E(t)* and τ at Different Contact Areas

This study investigates the relaxation modulus and time scales of triblock/diblock gels over a wide range of contact areas; macroscale (10s of mm^2), mesoscale (100s of μm^2) and microscale (~1 μm^2). The micelle diameters in Fig. 1a are on the order of nm, and thus the 100/0 triblock/diblock gels are expected to deform homogeneously over the lengthscales investigated here. Therefore, *E(t)*, τ and β should not depend on the method of testing considered in the present study. Macroscale, mesoscale, and microscale relaxation experiments showed that the instantaneous elastic modulus E_o of these gels was indeed independent of the method of interest. For example, the 100/0 triblock/diblock gel exhibited an instantaneous modulus of 95 kPa, 150±2 kPa and 63±0.7 kPa calculated at the macroscale, mesoscale and microscale, respectively, which are all within 50% variation. Further, differences between our macroscale rheometry and indentation results can be attributed in part to uncertainties in defining the contact point (for mesoscale experiments) and to the assumption of manufacturer-provided AFM probe diameter (in microscale experiments).

In contrast to this lengthscale-independence for *E(t)*, studies conducted in air on the 100/0 triblock/diblock gel showed that τ varied significantly among these methods. As the contact areas decreased, τ decreased by two orders of magnitude (τ_{AFM}= 36±2 s (in air) vs. $\tau_{macroscale}$= 2340 s (in air)). Because this gel is structurally homogeneous at the contact areas investigated here, the difference in the calculated τ values is attributed mainly to the increased contribution of adhesive forces to load relaxation that is observed at the small scales. As shown in the inset of Fig. 4a, visualization of contact between the flat punch AFM probe and polymer gel surface in air provided evidence of capillary flow of the gel along the probe sidewalls. Immersing the gel in water can decrease the capillary and electrostatic forces; however, water immersion did not decrease the total adhesion force for this system. In previous studies of stress relaxation of cells via AFM, authors have attempted to minimize adhesion by using chemically coated cantilevers, in order to make sure that the relaxation of the load is only due to viscoelastic nature of the material (Hiratsuka et al., 2009). We also attempted several such chemical functionalization approaches for these AFM probes, but did not identify a surface functionality that significantly reduced adhesion in air. Here we observe that minimization of probe-sample adhesion via addition of a surfactant to the immersion environment results in a calculated τ of 2050±388 s, which is comparable to macroscale results. Therefore, we conclude that in small scale stress relaxation experiments, adhesion should be accounted for and minimized to accurately determine relaxation times. The errors arising from the effect of adhesion will be even more crucial for accurate characterization of the mechanical properties of composite gels or soft tissues. In such structurally heterogeneous materials, the inferred mechanical properties will also depend on the volume of material probed.

5. CONCLUSION

In this study we report the relaxation modulus *E(t)* and characteristic time scales τ of triblock /diblock gels as a function of composition and lengthscales, and compare the results with that of heart tissue. We conclude that:

1. *E(t)* depends on vol-% triblock but does not depend on the volume of material probed over the lengthscales investigated.
2. Relaxation times calculated from experiments conducted in air do not depend on the vol-% triblock, but can depend on the volume of material probed due to relatively stronger contributions of adhesion at smaller lengthscales.
3. Relaxation time measured in air is two orders of magnitude smaller than relaxation times measured in PBS+0.2% Pluronic® F108, an immersion environment that minimizes probe-sample adhesion. Therefore, adhesion contributes significantly to load relaxation in small scale experiments, and must be taken into account in order to decouple adhesive and viscoelastic properties.
4. Heart tissue is more mechanically compliant and relaxes more quickly than the gels investigated here. Further optimization of these tissue surrogate gels could be achieved via manipulation of the gel composition by changing the chain length or the volume fraction of gel content.

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