Mixed Reversible Covalent Crosslink Kinetics Enable Precise, Hierarchical Mechanical Tuning of Hydrogel Networks

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Hydrogels play a central role in a number of medical applications and new research aims to engineer their mechanical properties to improve their capacity to mimic the functional dynamics of native tissues. This study shows hierarchical mechanical tuning of hydrogel networks by utilizing mixtures of kinetically distinct reversible covalent crosslinks. A methodology is described to precisely tune stress relaxation in PEG networks formed from mixtures of two different phenylboronic acid derivatives with unique diol complexation rates, 4-carboxyphenylboronic acid, and *o*-aminomethylphenylboronic acid. Gel relaxation time and the mechanical response to dynamic shear are exquisitely controlled by the relative concentrations of the phenylboronic acid derivatives. The differences observed in the crossover frequencies corresponding to pK_a differences in the phenylboronic acid derivatives directly connect the molecular kinetics of the reversible crosslinks to the macroscopic dynamic mechanical behavior. Mechanical tuning by mixing reversible covalent crosslinking kinetics is found to be independent of other attributes of network architecture, such as molecular weight between crosslinks.

Hydrogels from polymeric networks play a central role in a number of medical applications, including as cellular scaffoldings, drug delivery systems, soft tissue replacements, and wound dressings.[1–3] Precise engineering of hydrogel networks to produce mechanically defined biomaterials may improve their capacity to recapitulate or mimic the functional dynamics of native tissues.[4] In particular, the mechanical behavior of hydrogels under dynamic strain loading regimes are important factors influencing performance in cell scaffolding and as tissue replacement applications and must be tailored to suit specific tissues.[5–8] For example, stress relaxation in the extracellular matrix (ECM) is a key attribute of cell–ECM interactions and a important design parameter for hydrogels for tissue engineering.[9]

A variety of parameters control the physical properties and biological interactions of hydrogels materials and may ultimately dictate its success or failure.[10] Reversible chemical interactions have emerged as a useful crosslinking strategy to construct mechanically dynamic shear-thinning and self-healing hydrogels. A variety of transiently crosslinked systems have been demonstrated including those based on hydrogen bonding and electrostatic interactions as well as host–guest and receptor–ligand pairs.[11–13] Due to the reconfigurable nature of these interactions, dynamically crosslinked systems can exhibit stress relaxation and self-healing behavior. Those with shear-thinning behavior can be readily injected through high gauge medical syringes.[14] However, these systems have typically been designed from repeating weak interactions along the polymer backbone, which limits their mechanical performance. It is difficult to engineer mechanical properties in a precise fashion a priori and modifications inherently alter other properties of the network such as biochemical characteristics or effective mesh

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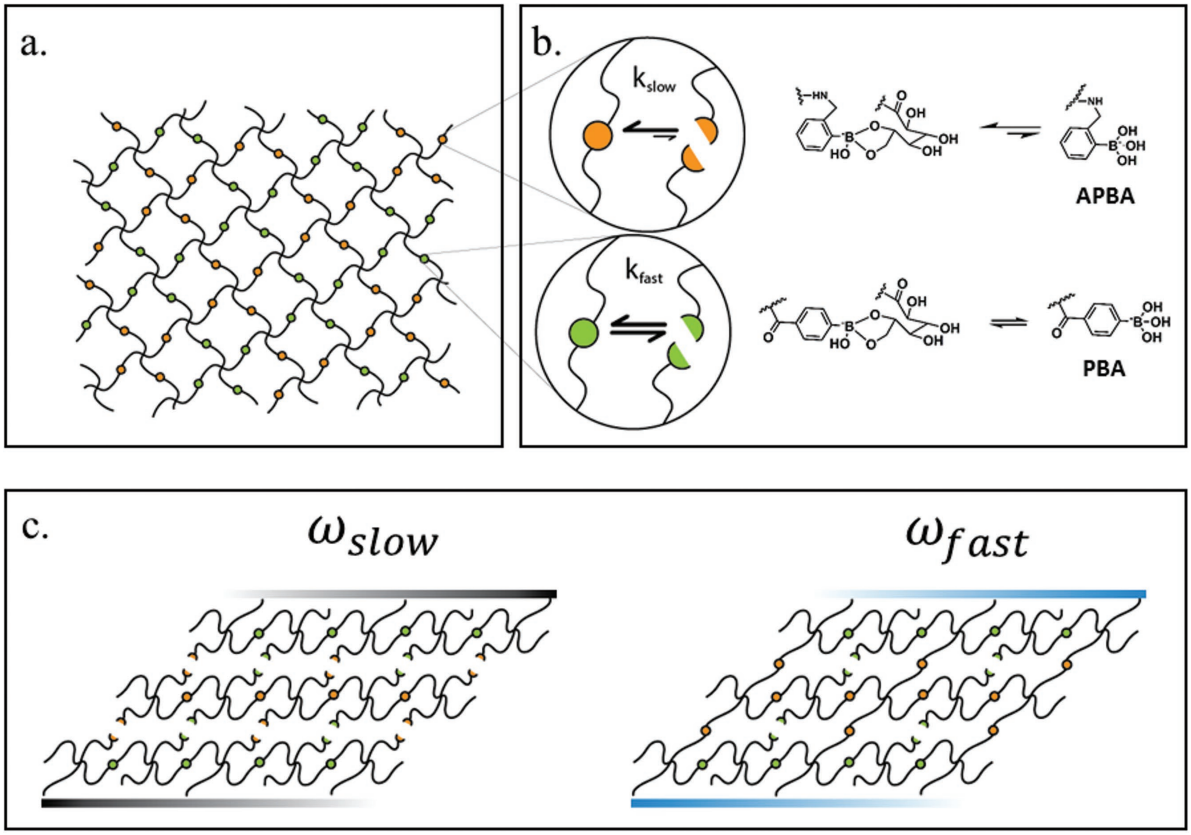

Figure 1. a) Schematic of hydrogels from 4-arm PEG macromers crosslinked by b) a mixture of reversible covalent complexes of different phenylboronic acid derivatives with *cis*-diols. c) Both slow and fast crosslinks behave as dissipation crosslinks for relatively slow strain rates, whereas only the fast dynamic crosslinks can dissipate stress for fast strain rates.

size. Recently, multiple metal coordination crosslinks with distinct kinetic rates were utilized to introduce multiple relaxation modes in viscoelastic hydrogel networks. This was a demonstration of decoupling the dynamic mechanical behavior from the backbone structure of a hydrogel network.[15] This orthogonal, hierarchical approach offers a promising strategy for the rational design of soft matter with multiple relaxation modes. Dynamic covalent crosslinks have also been explored to produce structurally dynamic polymers and polymer networks, and control relaxation timescales independent of other structural network attributes.[16,17] However, the a priori engineering of hydrogel networks with precise relaxation timescales, independent of the repeating polymer structure, remains a challenge. Herein, we describe a systemic approach to tuning the relaxation time in viscoelastic polymer networks using reversible covalent network linkages.

The reversible covalent complexation of phenylboronic acid (PBA) with *cis*-1,2 or *cis*-1,3-diol compounds has been investigated as a dynamic link to produce supramolecular structures and hydrogels.[18,19] Recently, it was reported that shear thinning and self-healing networks assembled from various phenylboronic acid and glucose-like diols can be developed for pH-sensitive delivery of therapeutic macromolecules.[20] Interestingly, in these studies, the location of the crossover frequency during dynamic rheology was sensitive to the pK_a of the PBA used. We hypothesize that precise, orthogonal tuning along a continuum of dissipation timescales can be achieved by forming networks containing multiple, kinetically distinct, PBA–diol pairs. Herein, we develop a methodology to precisely tune the mechanical properties of polyethyleneglycol (PEG) networks formed from mixtures of two different PBA derivates with unique diol complexation rates, 4-carboxyphenylboronic acid (PBA) and *o*-aminomethylphenylboronic acid (APBA) (**Figure 1** and Figure S1, Supporting Information).

4-arm PEG–PBA macromers were produced by either reacting 4-arm PEG–NH_2 (5–20 kDa) with *O*-(benzotriazol-1-yl)-*N*,*N*,*N*,*N*-tetramethyluroniumhexaflurophosphate (HBTU) activated 4-carboxyphenylboronic acid to produce PEG–PBA or by reductive amination of 4-arm PEG–NH_2 with 2-formylphenylboronic acid to produce PEG–APBA.[20] To produce the 4-arm PEG–diol macromers, D-glucolactone was reacted with 4-arm PEG–NH_2 in the presence of triethylamine. All gel formulations were produced by dissolving the PEG–PBA and PEG–diol macromers in buffered aqueous solution at 10 w/v%, and homogenized with vigorous mixing.

Five unique hydrogel formulations were produced by equimolar mixing of PEG–PBA and PEG–diol macromers, while varying the relative ratio of PBA:APBA macromers. For example in a 50:50 PBA:APBA gel, 50% of the phenylboronic

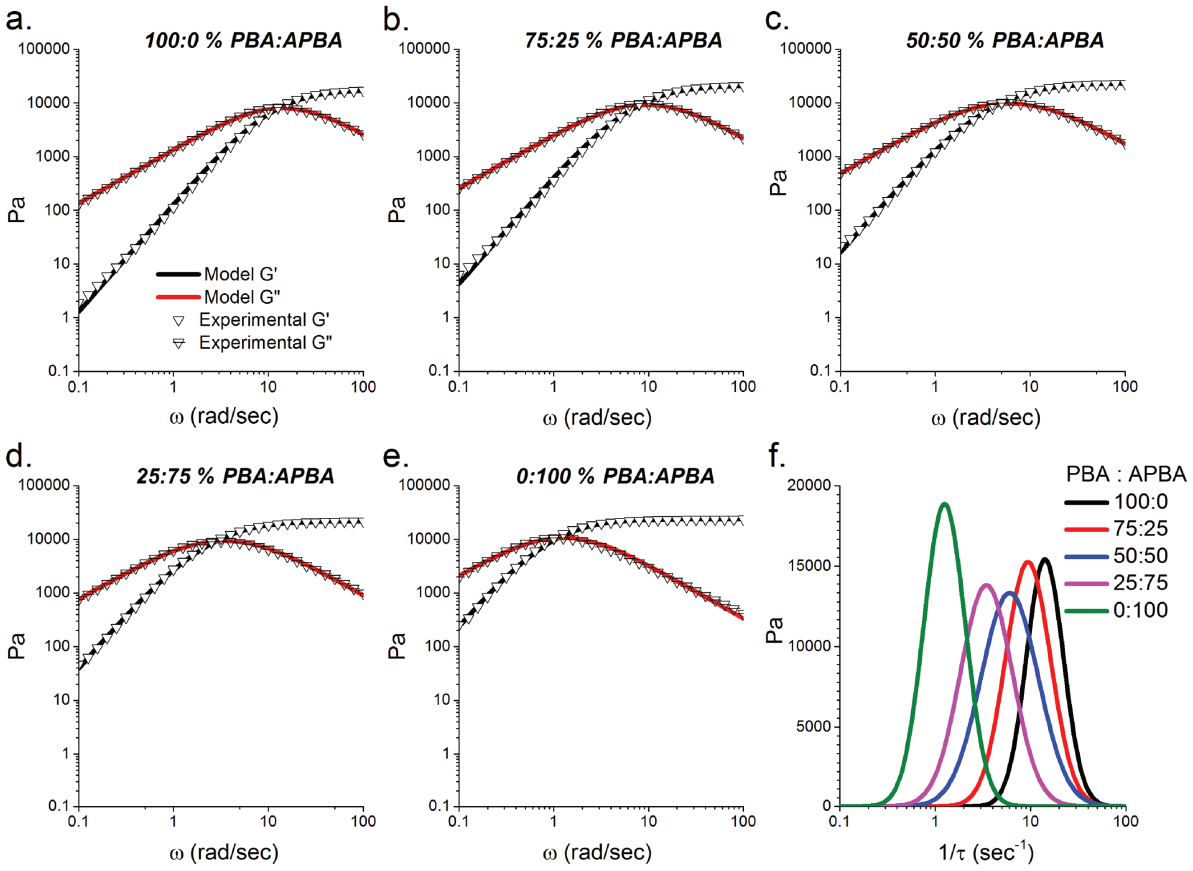

Figure 2. Rheological characterization of PBA:APBA mixtures. a–e) Oscillatory rheology measurements of PEG hydrogels (10 w/v%) from stoichiometric amounts of 5 kDa PEG–PBA and 5 kDa PEG–diol macromers formed at pH 7.4. The relative amounts of PBA to APBA were varied from 0:100% to 100:0%. Experimental rheology data (triangles) are overlaid with model rheology curves (solid lines). f) Infinite spectra for the mixtures in (a)–(e), from 0:100 PBA:APBA (green trace) to 100:0 PBA:APBA (black trace).

acid derivatives are PBA and 50% are APBA (Supporting Information). All mixtures exhibited viscoelastic network behavior, evidenced by the intersection of G' and G'' on frequency-sweep rheometry curves (**Figure 2**a–e). Owing to dynamic nature of the network crosslinks, the mechanical moduli observed in dynamic rheology are highly dependent upon the timescale of deformation: strain frequencies faster than the crosslink dynamics evoke an elastomeric response, while low frequency deformations allow stress dissipation as network junctions disengage. These characteristics are also true of entangled polymer networks, but the timescales of the mechanical response depend on structural factors such as persistence length and molecular weight, precluding hierarchical design.[21] In order to delineate the dissipation dynamics of the five mixtures, the rheological data were fit to a model of an infinite spectrum of Maxwell elements (Supporting Information), and the dissipation timescales were plotted for the various mixtures (Figure 2f).[22] The model assumes an infinite parallel arrangement of Maxwell elements (a compliance (capacitance) and a dashpot (resistance) in series). Each frequency location (ω) is represented as a Maxwell element with time constant τ, where $\tau = 1/\omega$. The model relaxation spectrum, $H(\tau)$, describes the entire distribution of Maxwell elements across timescales. The magnitude of $H(1/\omega)$ at a particular location is proportional to the energy dissipation at frequency ω. The area under the distribution, $\int_{-\infty}^{1/\omega} H(\tau)\mathrm{d}ln(\tau)$, is proportional to the energy storage at frequency ω and the plateau of G' from the rheology data. Mixtures of PBA and APBA have uniform, unimodal Maxwell distributions similar to their parent pure PBA or APBA gels, but with intermediate dissipation timescales. Strikingly, the dissipation timescale of PBA:APBA mixtures is exquisitely sensitive to the relative concentrations of PBA and APBA (Figure 2).

There were no significant differences in the elastic modulus (G' at the crossover frequency) or in the shape of the distribution of Maxwell elements for each of the mixtures, indicating that the accessible mechanical properties for each of the mixtures is the same, but that the time scales required to observe those properties is shifted, which we attribute to the differences in pK_a of the phenyl boronic acids. PBA has a higher reported pK_a than APBA, 7.8 and 6.5–6.7, respectively.[23–25] This is significant because it has been reported that the tetrahedral boronate anion is the predominant species that participates in PBA–diol complexation.[26] Therefore, the differences observed in the crossover frequencies

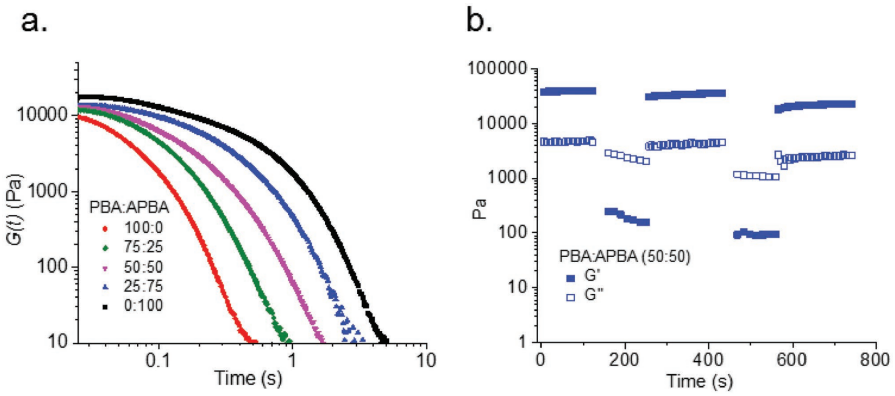

Figure 3. a) Relaxation plots of 10 w/v% gels formed at pH 7.4 from five different PBA:APBA mixtures (5 kDa), showing that relaxation timescale decreases with an increasing percentage of PBA ($\gamma = 10\%$). b) Self healing of 10 w/v% hydrogels formed from 50:50 mixtures of 5 kDa PBA:APBA reform robust hydrogels upon recovery from high strain ($\gamma = 500\%$).

corresponding to pK_a differences in phenylboronic derivatives directly connect the molecular kinetics of the reversible crosslinks to the macroscopic dynamic mechanical behavior. Gels formed entirely from PBA–diol interactions (100:0 PBA:APBA mixture) exhibit an order of magnitude shorter dissipation timescale compared to those of entirely APBA (0:100 PBA:APBA mixture) (Figure 2f). Consistent with these results were the decreasing exponential decay rates of the stress relaxation modulus for increasing APBA content, measured in the step strain paradigm (**Figure 3**a). The rate of stress relaxation is an important consideration for self-healing materials, especially in the context of injectable materials or dynamic cell culture systems: the rate of shear must be slower than the rate of the dynamic crosslinks. Previously, it was reported that 100:0 PBA:APBA gels are capable of self-healing, while 0:100 PBA:APBA does not self-heal when exposed to large strain.[20] Here we investigated the ability to self-heal in networks formed from APBA and PBA. We found that while 0:100 PBA:APBA did not exhibit self-healing, 50:50 PBA:APBA did self-heal rapidly after exposure to large strains (Figure 3b and Figure S2, Supporting Information).

We further investigated differences in the PBA–diol complexation kinetics between the phenylboronic acid crosslinks by measuring the mixtures' mechanical properties at various pH levels (**Figure 4**a–c). The pH is known to affect the balance between the bound and unbound states in PBA–diol complexes, with lower pH favoring the unbound state. The effect of pH is relative to the pK_a of the phenylboronic acid, since it is predominately the conjugate base of the phenylboronic acid, the phenylboronate anion, that complexes *cis*-diols and hydroxy acids.[26] Hydrogels formed from PBA–diol crosslinks are thus sensitive to pH changes, with low pH relative to the PBA pK_a resulting in faster self-healing and gradual flow under gravity.[18] By contrast, gels at high pH relative to the PBA pK_a have the characteristics of permanently crosslinked networks with limited ability to deform and self-heal. It was expected that increasing the pH would result in shifts of the dissipation timescales toward slower relaxation. For all PBA:APBA mixtures tested, there was at least an order of magnitude slower dissipation timescale at pH 8.5 compared to pH 7.4. For the 100:0 and 50:50 PBA:APBA mixtures, this temporal shift was

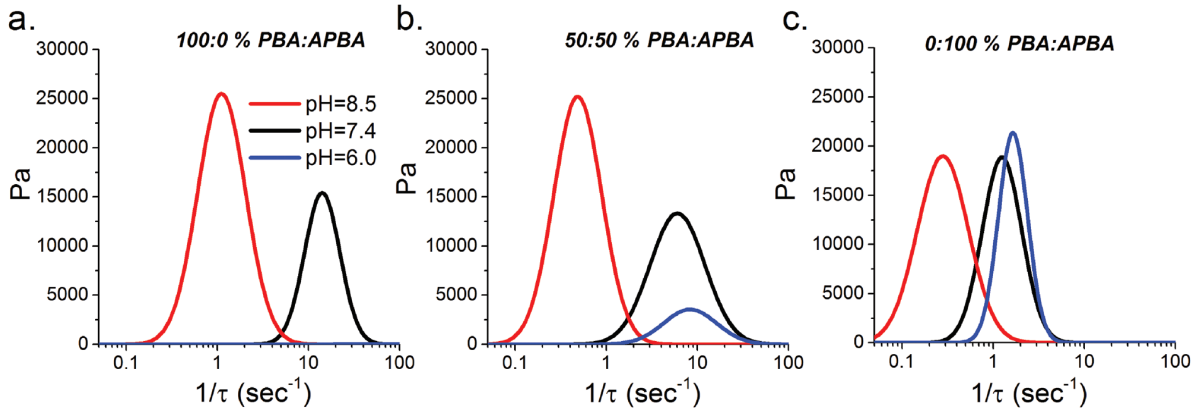

Figure 4. Effect of pH on relaxation spectra of hydrogels from 5 kDa polymers. a) 100:0 PBA:APBA hydrogels show a dramatic increase in modulus and relaxation timescale with increasing pH; with no gel observed at pH = 6.0. b) 50:50% PBA:APBA hydrogels also exhibit a notable increase in both modulus and relaxation timescale. c) Spectra for 0:100% PBA:APBA gels shift toward slower timescales at higher pH.

accompanied by a two fold increase in modulus (Figure 4a–c). The increase in elastic strength (G' at plateau, also reflected in the area under the Maxwell distribution curve) with increased pH indicates a higher crosslink density, and is consistent with a higher proportion of bound PBA–diol at elevated pH. The temporal shift is interpreted as a high pH induced shift in equilibrium constant favoring the tetrahedral boronate–diol complex and reducing the rate of formation of unbound boronate ion or boronic acid, thus reducing the rate of stress dissipation through crosslink cleavage. Interestingly, for the 0:100 PBA:APBA mixtures, there was a near order of magnitude increase in the dissipation timescale with increased pH, without a proportional increase in elastic strength. We interpret this as a decrease in the reverse complexation rate without a substantial increase in the proportion of bound complexes. This could be due to network heterogeneities caused by the observed rapid gelation that occurs at pH values far above the boronic acid pK_a. However, for 50:50 PBA:APBA gels, there was a significant decrease in the elastic energy stored, and a complete loss of elastic character in the 100:0 PBA:APBA accompanying decreases in pH.

To study the potential of this approach for tuning the dissipation timescales, we examined the effect of the average molecular weight between crosslinks (M_c). The M_c in this system is determined by the macromers size, thus we synthesized 5, 10, and 20 kDa 4-arm PEG macromers functionalized with PBA, APBA, or *cis*-like diol. The effect of increasing the M_c is to reduce the number of elastically active crosslinks (ν_e) per unit volume ($\overline{V}$). For elastomers, the relationship between shear modulus is directly proportional to the elastically active crosslink density[27]

$$G = kT\frac{\nu_e}{\overline{V}} \tag{1}$$

Thus, we expected that the gels formed from larger macromers would have a smaller elastic modulus, but that the dissipation timescales would be unaffected. Dynamic rheology of gels from 5, 10, and 20 kDa macromers reveals a reduction in ultimate elastic modulus with increasing molecular weight, without a shift in dissipation timescale for a given PBA:APBA mixture (**Figure 5**a–e). Importantly, the shift in timescales between mixtures was approximately uniform for the three molecular weights tested. These results demonstrate the

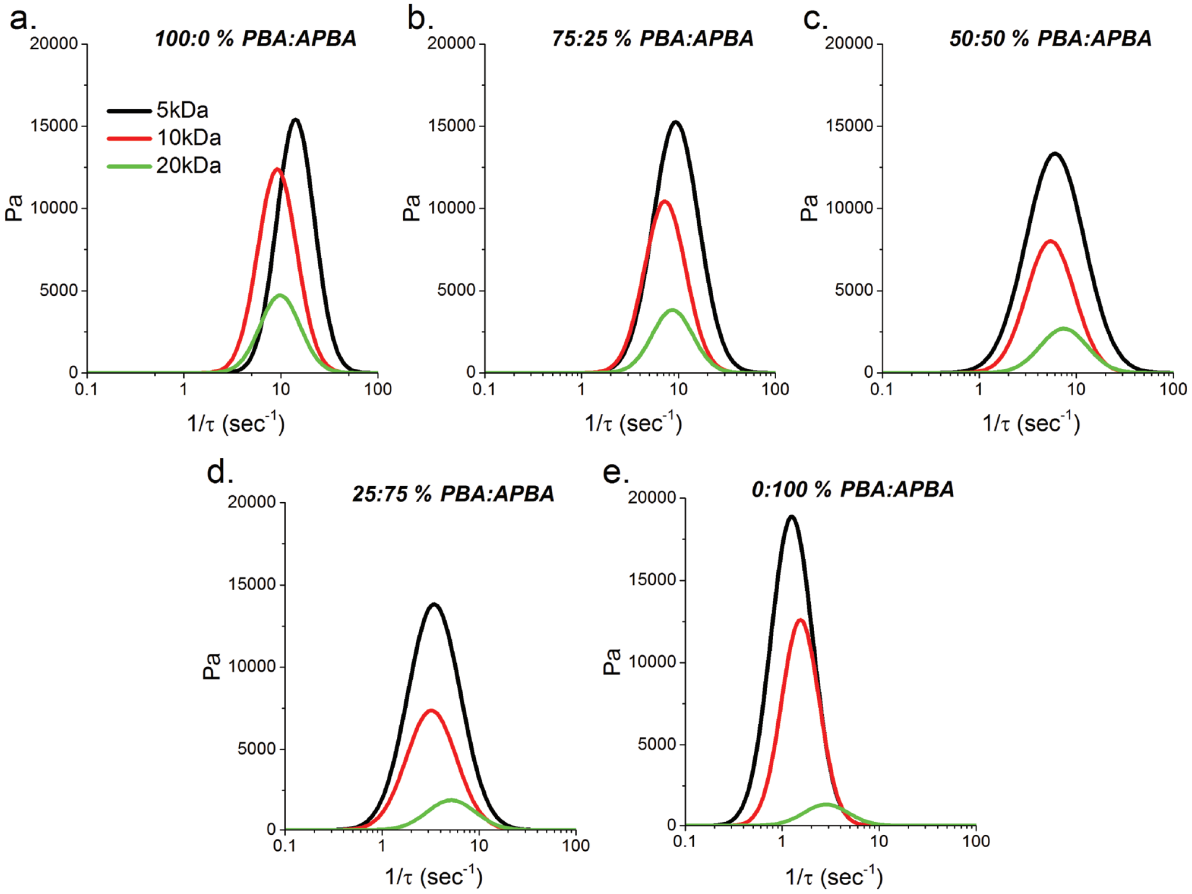

Figure 5. Independence of relaxation time scale and molecular weight. a–e) Relaxation spectra for PBA:APBA mixtures at pH = 7.4 for three different molecular weights: 5, 10, and 20 kDa phenylboronic acid and *cis*-diol macromers. Relaxation timescales increase with increasing percentage of APBA but are independent of macromers molecular weight. Increasing moduli are concomitant with decreasing macromers molecular weight, indicating an increase in active crosslink density.

orthogonality of the approach, showing that the tuning achieved in Figures 2f and 3a can be carried across other aspects of the network architecture.

In summary we have demonstrated the utility of mixtures of kinetically unique covalent crosslink dynamics in tuning the time-dependent mechanical response of bulk hydrogels. The self-healing nature of these systems makes them amenable for injectable clinical applications. This strategy builds on recent work aimed at hierarchical tuning of network dynamics in transiently crosslinked systems.[15] Important implications of tuning mechanical dynamics in hydrogels includes advanced 3D cell culture environments that more closely mimic native tissues.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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