

Internal Catalysis in Dynamic Hydrogels with Associative Thioester Cross-links

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ABSTRACT: Thioesters are an essential functional group in biosynthetic pathways, which has motivated their development as reactive handles in probes and peptide assembly. Thioester exchange is typically accelerated by catalysts or elevated pH. Here, we report the use of bifunctional aromatic thioesters as dynamic covalent cross-links in hydrogels, demonstrating that at physiologic pH in aqueous conditions, transthioesterification facilitates stress relaxation on the time scale of hundreds of seconds. We show that intramolecular hydrogen bonding is responsible for accelerated exchange, evident in both molecular kinetics and macromolecular stress relaxation. Drawing from concepts in the vitrimer literature, this system exemplifies how dynamic cross-links that exchange through an associative mechanism enable tunable stress relaxation without altering stiffness.

Polymers cross-linked with aqueous-compatible dynamic covalent bonds allow researchers to program dynamic behaviors into hydrogels with molecular tunability.^{1,2} Systems based on boronic ester exchange,³ hydrazone exchange,^{4–6} and thiol conjugate addition/elimination^{7,8} demonstrate how subtle structural modifications to the structure of dynamic covalent cross-links impart substantial changes in the resulting material's viscoelastic properties. By measuring the thermodynamic and kinetic parameters governing cross-link exchange, it is possible to predict macromolecular trends.^{5,7–10} Here, we report the use of aromatic thioesters as reversible dynamic covalent cross-links in hydrogels, highlighting how the associative exchange mechanism enables tunable stress relaxation without affecting matrix stiffness. We leverage internal catalysis through hydrogen bonding, rather than anchimeric assistance, to accelerate exchange without changing the exchange mechanism.¹¹

Of the dynamic covalent chemistries incorporated into dynamic hydrogels, the vast majority exchange through a *dissociative* mechanism, in which a cross-link first disengages via hydrolysis before re-condensing with the same or different partner. Condensation with a new partner permits the topological rearrangements responsible for macroscopic stress relaxation.¹² In dissociative networks, stiffness and stress relaxation are coupled to one another because the molecular rate processes giving rise to each property are interdependent.^{2,7,13} Stress relaxation is governed by the rate of cross-link dissociation (k_d), whereas stiffness is determined by the ratio of bound to unbound

cross-links, captured by the cross-link equilibrium constant K_{eq} (k_a/k_d). By tuning stress relaxation through the selection of binding partners with different k_d values, K_{eq} and thus stiffness are consequently affected.

Following reports by Anseth,^{14,15} Bowman,¹⁶ Grinstaff,^{17,18} and Sakai,¹⁹ we identified thioesters as a promising associative dynamic covalent cross-link to overcome the interdependence of stiffness and stress relaxation in viscoelastic hydrogels. In water, transthioesterification occurs via thiolate attack to form a tetrahedral intermediate,²⁰ allowing cross-link rearrangement to occur without a decrease in cross-link density. In associative systems, the molecular exchange rate k_{ex} correlates with stress relaxation timescale, while stiffness remains independent of the exchange chemistry. Therefore, cross-link density is instead controlled through the stoichiometry of reactive end groups during material fabrication.

Compared to their oxoester analogues, thioesters are a promising dynamic chemistry for aqueous systems at physiologic conditions because they are less stabilized by resonance and thus more reactive. In nature, the significance of thioesters is exemplified by coenzyme A.^{21–23} Synthetically, this increased reactivity underlies the utility of thioesters for methods like native chemical ligation.^{24,25} Interestingly, while thioesters react with nucleophiles like amines and carbanions far faster than oxoesters do, their rates of hydrolysis remain similar.^{20,26,27}

Previous reports of hydrogels fabricated with linear, aliphatic thioester-based crosslinks demonstrate stress relaxation on the order of 10^4 – 10^6 seconds (**Figure 1**).^{14,15}

Stress relaxation was accelerated by raising the pH, using larger excess of free thiol, or adding a thiophenol catalyst. In order to match the viscoelastic time scales of soft tissues, which undergo stress relaxation on the order of 10^1 – 10^3 s (Table S3),^{28,29} materials that exhibit faster rearrangement are needed. This gap motivated us to develop thioester cross-links with faster exchange rates.

We incorporated two key design features to accelerate dynamic exchange: 1) an aromatic, rather than aliphatic, acyl scaffold and 2) internal catalysis through a hydroxyl group positioned *ortho* to the thioester (Figure 1). Internal catalysis emerges when a dynamic bond and catalytic moiety are covalently tethered within the same polymer matrix; though the catalyst/dynamic bond ratio is 1:1, the catalytic site can support multiple turnovers without being consumed.^{11,32} Moreover, the proximity between the dynamic bond and the catalytic site lowers the entropic cost of the transition state and allows weak acids and bases to be effective catalysts. The H-bonding strategy applied here maintains the associative nature of exchange, whereas neighboring group participation through covalent interactions, like intramolecular annulations, can result in a topologically dissociative exchange.^{33,34} While benzoyl esters are generally considered less reactive than acetyl esters due to resonance stabilization,^{30,31} we found that benzoyl thioesters underwent faster exchange than their aliphatic counterparts.

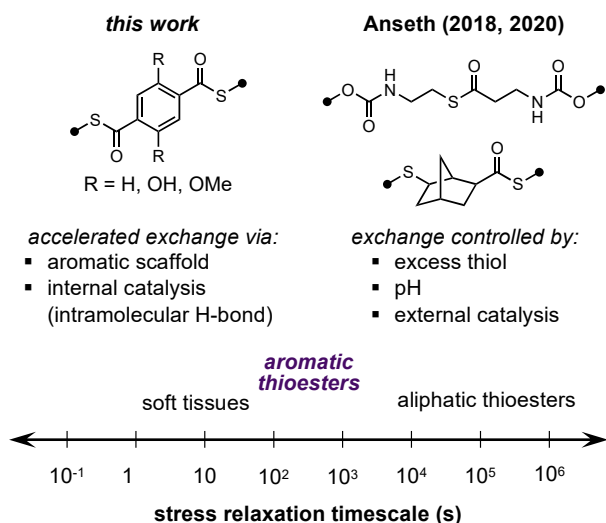


Figure 1. Comparison of stress relaxation timescales between native soft tissues and synthetic covalent adaptable hydrogels containing dynamic thioester cross-links. In this work, aromatic thioester cross-links are designed to accelerate aqueous exchange, leading to hydrogels with stress relaxation timescales matching those of liver, muscle, and skin.

We first devised a small-molecule model system to measure the kinetics of transthioesterification (k_{ex}) for model substrates using high-performance liquid chromatography (HPLC). We synthesized three S-alkyl benzothioate derivatives (abbreviated **ArT**), substituting the *ortho* position of the aromatic group with either a hydrogen, a hydroxyl group, or a methoxy group. As depicted in Figure 2a, the **ArT_{BT}** starting compounds were subjected to thiol exchange with a chemically distinct thiol **ET** under

pseudo-first order conditions, varying the stoichiometric ratio between 10–200 equivalents. The concentrations of **ArT_{BT}** and the exchange product **ArT_{ET}** were monitored over time by high-performance liquid chromatography (HPLC) based on their UV-Vis absorbance relative to an internal standard. Slopes of the pseudo-first-order kinetic profiles (see Supporting Information (SI), Figure S3-S5), which represent k_{obs} , were plotted against the concentration of **ET** to obtain k_{ex} for each substrate.

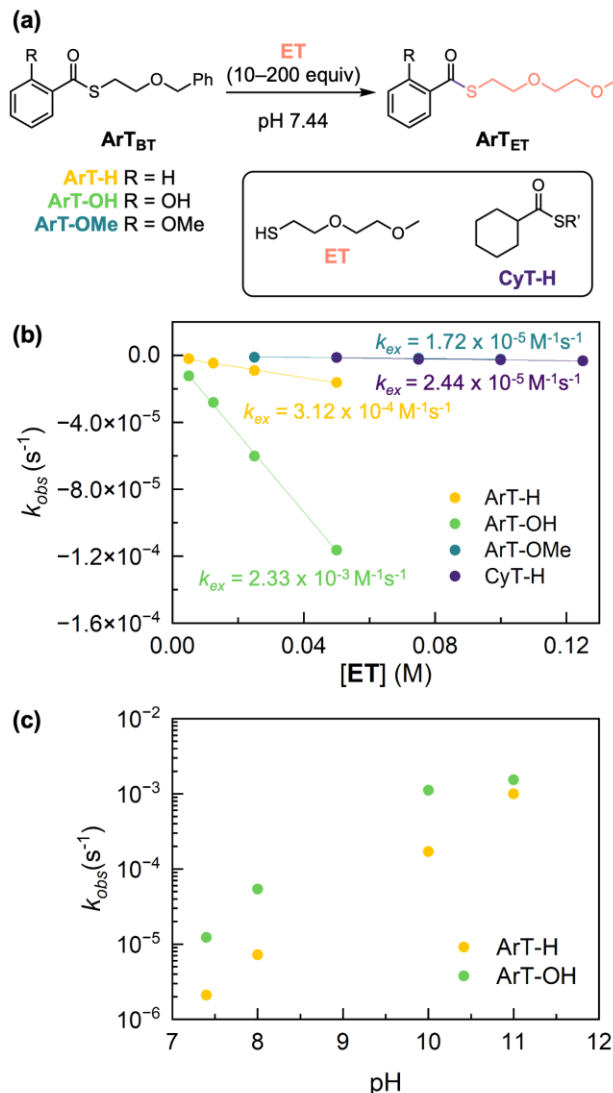


Figure 2. (a) Small-molecule transthioesterification reaction used to determine rates of dynamic exchange with **ArT-H**, **ArT-OH**, and **ArT-OMe**. (b) Observed rate constants (k_{obs}) were determined for the model reaction, varying the stoichiometric ratio of **ET** to substrate between 10–100 equiv (**ArT-H**, **ArT-OH**), 50–200 equiv (**ArT-OMe**), or 100–250 equiv (**CyT-H**). The slope of each profile represents k_{ex} for each substrate in 6:4 aqueous HEPES (0.1 M, pH 7.44)–acetonitrile. (c) k_{obs} versus pH for **ArT-H** and **ArT-OH**.

These experimental kinetic studies demonstrated that **ArT-OH** exhibits accelerated exchange relative to **ArT-H**, an effect that is mitigated when internal hydrogen-bonding is blocked in **ArT-OMe**. The pseudo-first order rate constant for exchange with **ArT-OH** was $2.33 \times 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$, a

~7.5x rate enhancement compared to k_{ex} for **ArT-H**, which was $3.12 \times 10^{-4} \text{ M}^{-1} \text{ s}^{-1}$ (**Figure 2b**). Conversely, k_{ex} for **ArT-OMe** was determined to be $1.72 \times 10^{-5} \text{ M}^{-1} \text{ s}^{-1}$, an order of magnitude slower than **ArT-H** (**Figure 2b**). We attribute this deceleration to resonance electron donation from the methoxy substituent into the thioester carbonyl π system, which renders it less electrophilic compared to **ArT-H**. Control experiments using phenol as an exogenous catalyst with **ArT-H** did not exhibit a significant change in k_{ex} relative to **ArT-H** alone (**Figure S8**). This finding indicates that the observed rate acceleration of **ArT-OH** relative to **ArT-H** can be ascribed to a proximity effect. To demonstrate the effect of the aromatic ring, we synthesized **CyT-H**, an aliphatic thioester, for which k_{ex} was determined to be $2.44 \times 10^{-5} \text{ M}^{-1} \text{ s}^{-1}$ (**Figure S6**). Therefore, the exchange rates offered by these aromatic thioesters spans two orders of magnitude, and the aromatic thioesters **ArT-H** and **ArT-OH** exchange faster than aliphatic **CyT-H**. We did not observe hydrolysis of the small molecule substrates over the course of the reactions (**Figure S7**).

We used density functional theory (DFT) to calculate energy-minimized structures for the three substrates and their anionic tetrahedral intermediates following the addition of methanethiolate in an implicit water environment with the conductor-like polarizable continuum model (**Figure 3a**).³⁵ Previous work has shown that under aqueous conditions, thiolate, not neutral thiol, is the primary nucleophilic species.^{20,36} The lowest-energy conformer of each tetrahedral intermediate was used as a starting point to obtain transition state geometries and calculate energy barriers for thiolate addition. The lowest-energy conformers involving **ArT-OH** in the substrate, transition state, and tetrahedral intermediate all feature intramolecular hydrogen bonding between the *ortho* hydroxyl group and the carbonyl group (**Figure 3b**), leading to the lowering of the transition state barrier to 19.4 kcal/mol when compared to **ArT-H** and **ArT-OMe**, which have barriers of 21.0 kcal/mol and 22.0 kcal/mol, respectively. To further support the hypothesis that internal hydrogen bonding promotes the reactivity of **ArT-OH**, we constrained the dihedral angle of the hydroxyl group to avoid internal H-bonding both in the ground and transition states, leading to the increase in the calculated barrier by 3.1 kcal/mol (22.5 kcal/mol, **Figure 3b**), which is comparable to the barrier with **ArT-OMe**, consistent with the similar electron-donating effects of these two groups. The calculated $\Delta\Delta G^\ddagger$ values correlated with those derived from experimental relative rates (**Figure 3c**).

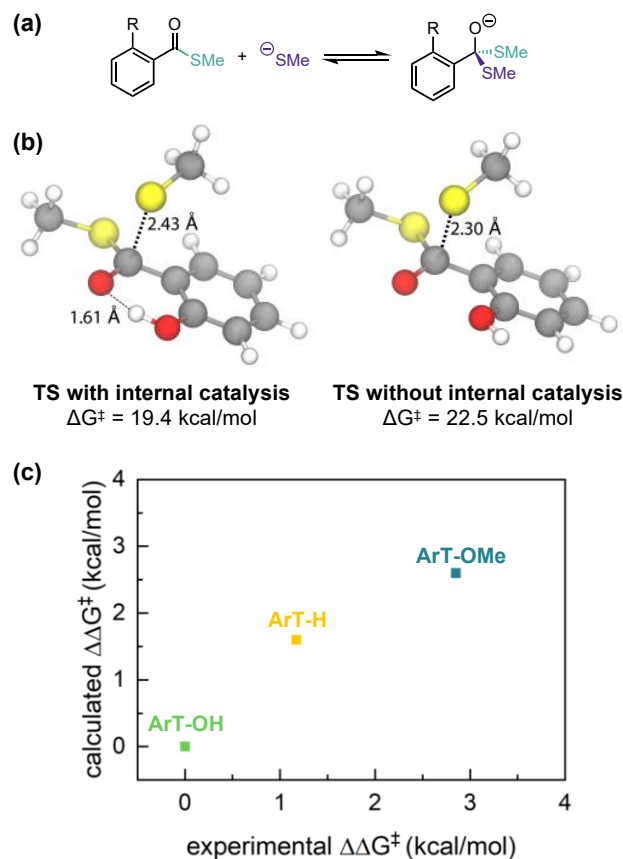


Figure 3. (a) Methanethiolate addition step used to calculate energy-minimized structures and tetrahedral intermediates for all aromatic thioester substrates. (b) Transition states for methanethiolate to **ArT-OH** with and without internal catalysis. (c) Correlation between calculated and experimental $\Delta G^\ddagger$ normalized to **ArT-OH**.

To experimentally support the hypothesis that accelerated exchange arises from internal hydrogen bonding, we measured pseudo-first order rate constants for **ArT-H** and **ArT-OH** at three additional pH values (**Figure 2c**). At pH values of 8.0 and 10, exchange for both thioesters is accelerated, as expected based on the higher concentration of thiolate, but the H-bonding effect remains: **ArT-OH** exchange is 7.5 and 6.5 times faster than that of **ArT-H**, respectively. At pH 11, however, the rate enhancement decreases to just 1.5 (tabulated data available in **Table S1**). We determined the pKa of **ArT-OH** to be ~9.9 through titration (**Figure S9**), suggesting that at higher pH, the electrophile exists mostly in its deprotonated phenolate form. Consequent loss of the intramolecular hydrogen bond therefore results in similar rates for the **ArT-H** and **ArT-OH** substrates. To determine if base-catalyzed hydrolysis of the thioesters was significant at elevated pH values, we ran kinetics on a sample of **ArT-OH** in 6:4 aqueous HEPES buffer (0.1 M, pH 11)–acetonitrile without **ET**, yielding k_{obs} for hydrolysis on the order of 10^{-6} , 3 orders of magnitude slower than the k_{obs} of exchange. Therefore, we concluded that hydrolysis was not significant under these conditions.

We next synthesized bifunctional aromatic thioester cross-linkers bearing norbornenes and fabricated stress-relaxing hydrogels (**Figure 4a**). Mercapto-terminated 4-

arm poly(ethylene glycol) (5 kg/mol) was cross-linked with the norbornene cross-linkers via a photoinitiated thiol-ene reaction in the presence of eosin Y and 525 nm green light (see SI for details). We targeted a thiol to norbornene molar ratio of 2:1, leaving free thiols to participate in trans-thioesterification. To bypass the poor solubility of the thioester cross-links in aqueous buffers, we first formed the network in dichloromethane, which solubilizes all three components (PEG, Eosin Y photoinitiator, and cross-linker), then dried and reswelled the network in aqueous HEPES buffer (0.1 M, pH 7.44). Because networks are formed in solvent, it was not possible to monitor crosslinking *in situ* by FTIR, but dried networks show evidence of increasing norbornene conversion with increasing irradiation time (Figure S10). This method also allows these networks to be characterized in other buffer formulations and at other pH values.

We characterized the gels with oscillatory shear rheology, demonstrating that all gels exhibit similar plateau moduli (1500 ± 150 Pa) but different stress relaxation time scales according to the trends observed in small-molecule kinetics studies. Frequency sweeps of the gels showed overlapping storage moduli at higher frequencies; none of

the cross-linkers underwent sufficiently fast exchange to observe a crossover point within the experimentally accessible range (Figure 4b). Fitting normalized stress relaxation data to a single-element Maxwell model yielded characteristic relaxation times (τ) of 170, 505, 531, and 1182 s for **gel_{ArT-OH}**, **gel_{ArT-H}**, **gel_{ArT-OMe}**, and **gel_{CyT-H}** respectively. The rates of stress relaxation qualitatively follow the trends in exchange rate extracted from small molecule kinetics studies. Therefore, accelerating trans-thioesterification through internal hydrogen bonding also leads to faster stress relaxation. Quantitatively, however, a plot directly comparing $\log(\tau^{-1})$ to $\log(k_{ex})$ indicated that the stress relaxation of the hydrogels is less sensitive to substituent effects compared to the small-molecule exchange rates (Figure S9). For example, stress relaxation for **gel_{ArT-OH}** is only ~3-fold faster than that of **gel_{ArT-H}**, compared to the 7.5-fold difference in small-molecule exchange rates. Moreover, even though **CyT-H** underwent exchange ~1.4 times faster than **ArT-OMe** in small-molecule kinetics, **gel_{ArT-OMe}** underwent stress relaxation ~1.8 times faster than **gel_{CyT-H}**. We have previously shown that $\log(\tau^{-1})$ and $\log(k_{ex})$ are directly proportional for another associative dynamic cross-link, dithioalkylidene conjugate acceptors.⁸

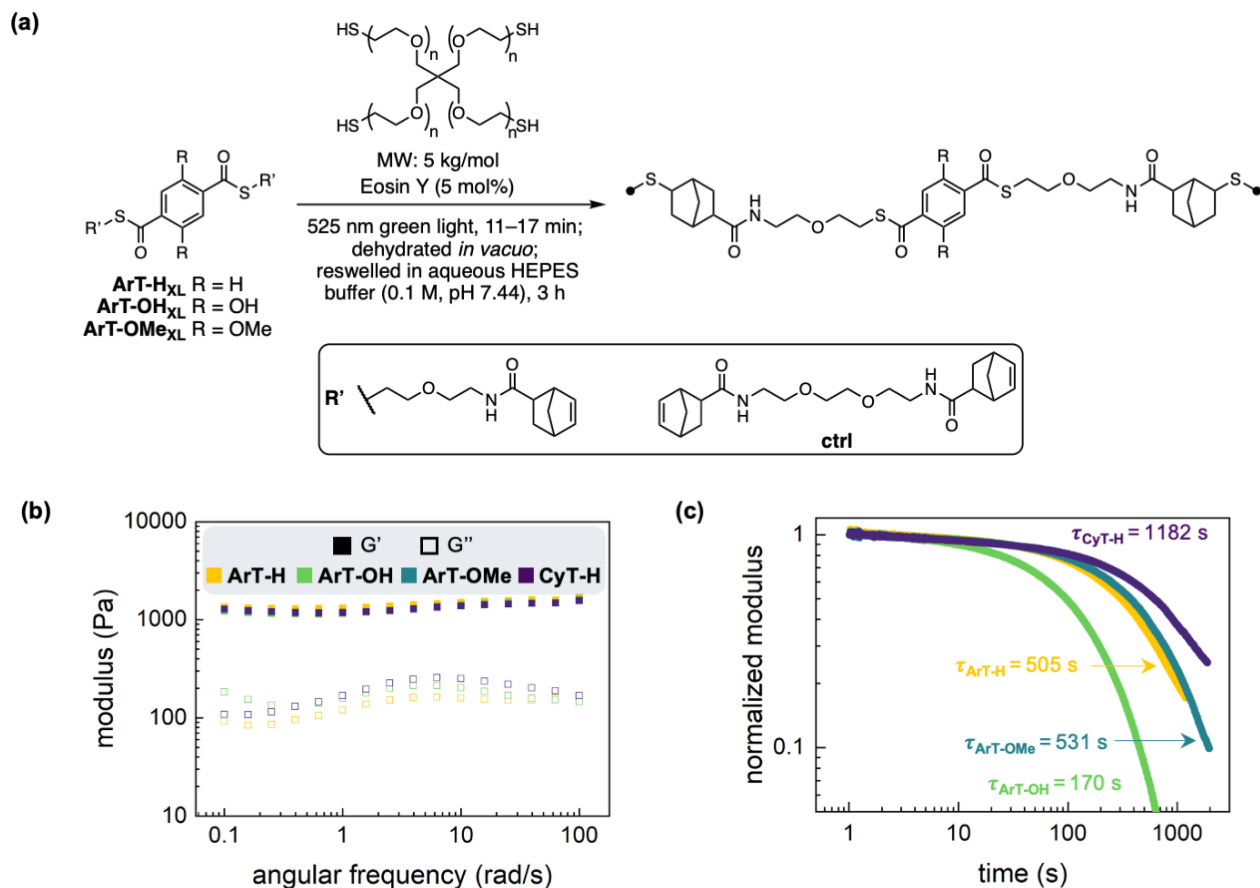


Figure 4. (a) Conditions for network formation by photoinitiated thiol-ene reaction with 4-arm mercapto-terminated PEG (5 kg/mol) and bifunctional norbornene cross-linkers. (b) Representative frequency sweeps of **gel_{ArT-H}**, **gel_{ArT-OH}**, **gel_{ArT-OMe}**, and **gel_{CyT-H}** in HEPES (10 w/v%, 0.1 M, pH 7.44, 5% strain). (c) Stress relaxation profiles of **gel_{ArT-H}**, **gel_{ArT-OH}**, **gel_{ArT-OMe}**, and **gel_{CyT-H}** (5% strain). τ values were obtained by fitting data to a single-element Maxwell model and taking the average of 3 datasets.

We hypothesize that this discrepancy may be explained by the relatively slow rate of thioester exchange, which allows other factors to influence stress relaxation. Previously, we determined that hydrogels fabricated using the procedure in **Figure 4a** with a non-exchanging control crosslinker (**ctrl**) exhibit a τ value of 1553 s despite the absence of dynamic bonds,⁸ which we attributed to reptation from dangling ends.^{37,38} The thioester hydrogels relax stress within an order of magnitude of the control **gel-ctrl**. If network relaxation is significantly influenced by network topology and dynamics, we cannot ascribe viscoelastic properties solely to molecular exchange. In addition, the small-molecule model systems we studied kinetically are not identical to the bifunctional cross-linkers: the cross-linkers contain an additional *para* ester and *meta* hydroxy or methoxy group that could perturb the electronics of the electrophilic site and thus the exchange rate of the cross-linkers. Nevertheless, the trends in **Figure 4** support the accelerating effect of an aromatic core and internal H-bonding for thioester exchange. As expected, these hydrogels are qualitatively self-healing (Figure S25). We also attempted to quantify self-healing rates using high-strain experiments,³⁹ but the sample was ejected from the rheometer during the strain ramp.

In conclusion, we have shown that aromatic thioester cross-links yield stress-relaxing hydrogels that undergo dynamic rearrangement under aqueous conditions at physiologic pH. These hydrogels exhibit stress relaxation on the order of 10^2 – 10^3 s, similar to soft tissues, and trends correlate qualitatively with small-molecule exchange kinetics. We acknowledge that these materials do not recapitulate all aspects of soft tissues, such as strain stiffening⁴⁰ and fibrillarity.⁴¹ Further modifications, such as the incorporation of cell-adhesive ligands, will be required to employ these hydrogels as a viable matrix for cell culture. This study also contributes to a growing body of literature demonstrating that physical organic concepts commonly used to rationalize chemical reactivity in small-molecule reactions can be used to predictively control macromolecular properties.^{42,43,2} We envision that the continued development of structure–reactivity–property relationships will enable the design of hydrogels with tailored viscoelasticity and responsiveness to external stimuli such as light.^{44–47}

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Materials and methods, experimental procedures, kinetic data, NMR and UV-vis spectroscopy, rheological data, computational details (PDF)
xyz coordinates of calculated structures (xyz)
summary of calculated energies (xlsx)

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Notes

The authors declare no competing financial interest.

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