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Structure and Mechanical Response of Protein Hydrogels Reinforced by Block Copolymer Self-Assembly

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Abstract

A strategy for responsively toughening an injectable protein hydrogel has been implemented by incorporating an associative protein as the midblock in triblock copolymers with thermoresponsive poly(N-isopropylacrylamide) (PNIPAM) endblocks, producing materials with a low yield stress necessary for injectability and durability required for load-bearing applications post-injection. Responsive reinforcement triggered by PNIPAM association leads to significant increases in the gel's elastic modulus as well as its resistance to creep. The performance of these materials is a strong function of molecular design, with certain formulations reaching elastic moduli of up to 130 kPa, effectively reinforced by a factor of 14 over their low temperature moduli, and having stress relaxation times increased by up to a factor of 50. The nanostructural origins of these thermoresponsive enhancements were explored, demonstrating that large micellar cores, high PNIPAM volume fractions, and high densities of associating groups in the protein corona lead to the greatest reinforcement of the gel's elastic modulus. Gels with the largest micelles and the highest packing fractions also had the longest relaxation times in the reinforced state. These combined structure and mechanics studies reveal that control of both the micellar and protein networks is critical for making high performance gels relevant for biomedical applications.

Introduction

Shear thinning hydrogels have emerged as an important technology for the preparation of injectable matrices for tissue engineering, regenerative medicine, and drug delivery,^{1–4} where minimally invasive methods of implanting gels promise faster patient recovery and lower medical costs. Compared with other injectable biomaterials, shear thinning hydrogels are attractive because they can be formulated as stable gels *ex vivo* and will rapidly self-heal on the order of seconds following injection.^{5–8} Shear thinning hydrogels prepared from artificially engineered protein polymers are particularly advantageous because they can be easily decorated with cell-signalling domains and protease sites using genetic engineering,⁹ allowing for better integration of the implanted matrix and control of cell behaviour within the matrix.¹⁰ Artificially engineered telechelic proteins with coiled-coil self-associating domains joined by a soluble linker domain have been shown to form model shear thinning

physical hydrogels,^{11, 12} where network structure and relaxation dynamics can be controlled through precise manipulation of the polypeptide sequence.^{13–15} These gels have been studied as injectable delivery vehicles due to their shear banding behaviour that enables a high survival rate of encapsulated cells after injection.^{11, 16, 17} However, as with most other shear thinning hydrogels, the transient physical crosslinks that impart a low yield stress required for clinical use also make the materials susceptible to both mechanical failure and rapid erosion when introduced into a biological setting.^{18, 19}

Double networks have emerged as a powerful approach to reinforce hydrogels, resulting in substantial improvements in fracture toughness²⁰ and extensibility.²¹ The concept of synergistically employing independent networks within hydrated materials has been adopted successfully in a wide variety of synthetic systems including interpenetrating networks in chemically crosslinked gels^{21–26} and micellar networks with chemically crosslinked coronae.²⁷ An alternative toughening strategy utilized in nanocomposites involves the dispersion of small amounts of stiff, high aspect ratio nanoparticles in a soft matrix, which can also yield large improvements in toughness and extensibility.^{28–30} In addition, double network gels crosslinked by purely physical interactions have been prepared using multiple orthogonal, reversible associating moieties.^{31, 32} Incorporating networks of physical associations into such gels is particularly advantageous because it provides the sacrificial bonds needed for energy dissipation, crack blunting, and enhanced overall toughness, while allowing for self-healing and fatigue resistance.³³

To achieve shear thinning and high toughness in an injectable gel, the material must be responsively reinforced to transform from a yielding state during injection to a toughened state after implantation into the body. To address this challenge, we recently developed materials that thermoresponsively form a second orthogonal physical network within shear thinning protein hydrogels.³² Poly(N-isopropylacrylamide) (PNIPAM), which exhibits lower critical solution behaviour in water around 32°C, was conjugated near the N- and C-termini of an artificial associative protein to form a triblock copolymer. This double physical network hydrogel incorporates two independent associating functionalities in a single macromolecule: self-associating α -helical domains form the primary network that is active at temperatures up to 50°C, while endblock aggregation above roughly 20°C forms the second reinforcing network (Figure 1).

The presence of orthogonal, reversible associations on two separate length scales introduces complexity into these double network systems that can influence both the thermodynamics of self-assembly and the gel's mechanical response. The phase behaviour of polymers decorated with low valency associating groups is controlled by excluded volume interactions and the association density along the chain; even in a good solvent for the backbone chain, a high density of strong associations can lead to phase separation due to exchange entropy of the associating groups.^{34, 35} Classical transient network theory demonstrates that the high frequency elastic modulus of these gels is directly proportional to the number density of elastically effective bridging chains,^{36, 37} which in turn depends on gel concentration and network topology.³⁸ In contrast, telechelic polymer gels are better described as a condensed phase of interacting micelles,^{39, 40} where excluded volume interactions and entanglements among corona chains contribute along with the entropic elasticity of bridging chains and

hydrophobe disengagement to control the static and dynamic mechanical properties of the gel.^{39, 41} Hybrid triblock copolymers containing self-associating helices in the midblock incorporate one network of each type.

The ability to manipulate various aspects of the molecular architecture of these polymers—through genetic engineering of the protein midblock and controlled free radical polymerization of the synthetic endblocks—provides the opportunity to understand the interactions between both networks that control the gel's nanostructure and mechanical properties. Previously, a single hybrid block copolymer with this polymer-protein-polymer architecture was shown to form a shear thinning hydrogel that could be responsively reinforced, demonstrating large increases in elastic modulus and shear yield stress, as well as significant decreases in the erosion rate and creep compliance at elevated temperatures. In this work, block copolymers with a range of block fractions, midblock lengths, and numbers of associations in the midblocks are synthesized, and their temperature-dependent structure and mechanics are studied in detail to understand the fundamental structural origins of these enhanced mechanical properties. The presence of midblock associations within triblock copolymers has a strong influence on self-assembly in these gels, shifting phase boundaries to lower temperatures and lower concentrations than are otherwise expected. In the self-assembled state, these midblock associations act to stiffen the micellar coronae, making large contributions to the high temperature modulus. Maximum reinforcement can be achieved when the size and volume fraction of micelles in the self-assembled network are maximized, leading to gels with over an order of magnitude improvement in stiffness and reduction of creep compliance at physiologically relevant temperatures.

Experimental Methods

Materials and Synthesis

Hybrid block copolymers were prepared from artificially engineered associative proteins and monodisperse PNIPAM as described previously (Figure 1).³² Briefly, associative proteins with thiol functionalities at both ends were produced biosynthetically in *Escherichia coli* and purified from clarified, nuclease-treated cell lysates by ammonium sulfate precipitation. Genes encoding for each protein were engineered as repetitive, alternating fusions of sequences for soluble polyelectrolyte linker domains (C₁₀)⁴² and pentameric α -helical associating domains (P),^{19, 43} flanked by two cysteine residues. PNIPAM was synthesized via Reversible Addition Fragmentation Chain Transfer (RAFT) polymerization with a single maleimide functionality incorporated in the polymer endgroup.⁴⁴ Proteins were reacted overnight at 4 °C with a 10-fold molar excess of PNIPAM at pH = 8.0 in the presence of a 10-fold molar excess of tris(2-carboxyethyl) phosphine (TCEP) per thiol. Unreacted PNIPAM was removed by anion exchange chromatography over QAE Sephadex (A-50) resin (GE Healthcare) under denaturing conditions at 4°C. To manipulate the triblock:diblock ratio in gels, a version of the C₁₀(PC₁₀)₄ protein was synthesized containing a single thiol functionality at the C-terminus. The same molecular weight of PNIPAM was conjugated to C₁₀(PC₁₀)₄ with either one or two thiols per protein, and unreacted protein was removed by two rounds of thermal precipitation at 37°C in 6 M urea and 2 M sodium chloride prior to anion exchange

chromatography. A series of gels were prepared with controlled triblock:diblock ratios by blending difunctional and monofunctional conjugates.

Protein molecular weights and purity were assessed by MALDI-TOF (Table S1) and SDS-PAGE (Figure S1). PNIPAM ($dn/dc = 0.0761 \pm 0.0007 \text{ mL g}^{-1}$ at 25°C , $\lambda = 658.0 \text{ nm}$) molecular weight distributions were characterized in DMF with an Agilent 1260 GPC system using three ResiPore columns, a Wyatt Mini-DAWN TREOS 3-angle light scattering detector, and a Wyatt Optilab T-rEX refractive index detector (Figure S2). Conjugation efficiency was estimated for each material by densitometry via SDS-PAGE using Bio-Rad Image Lab software.

Small Angle Neutron Scattering (SANS)

SANS experiments examining gel nanostructure as a function of midblock design and endblock molecular weight were performed at the Low-Q Diffractometer (LQD) at the Lujan Center at Los Alamos National Laboratory, and controlled endblock conversion studies with fine temperature sampling were performed at the NG-7 beamline at the NIST Center for Neutron Research (NCNR).⁴⁵ Samples were prepared in deuterated buffers at the specified w/v concentrations, and swollen at 4°C for at least 24 hours until homogenous. The gels were sealed into 0.5 mm thick sample cells with quartz windows. At the NCNR, scattering patterns were collected at three sample-detector distances (1 m, 6 m, and 13 m) at regular temperature intervals. Samples were equilibrated at least 30 minutes at each temperature. Data collected at Lujan and the NCNR were reduced and converted to absolute units according to established procedures.^{46, 47} The data were fit to the Percus-Yevick disordered hard-sphere model⁴⁸ with a lognormal polydispersity distribution function, as described previously.³²

Depolarized Light Scattering (DPLS)

DPLS^{49, 50} measurements were taken using a linearly polarized 662 nm laser. Gels were hydrated in 100 mM phosphate buffer (pH = 7.6) at 4°C until homogenous and transparent, typically 24–48 hours, and sealed in quartz sample cells with a 1 mm thick teflon spacer with a 3 mm bore. Samples were heated and cooled at $1^\circ\text{C}/\text{min}$ over the temperature range $5\text{--}50^\circ\text{C}$.

Differential Scanning Calorimetry (DSC)

DSC was performed on a TA Instruments Q-10. Gels were loaded into hermetically sealed aluminium pans and cycled twice at $5^\circ\text{C}/\text{min}$ over the range $5\text{--}50^\circ\text{C}$. Transition temperatures (T_i) were determined from the peak position on the second cycle.

Rheology

Oscillatory shear rheology and creep measurements were performed on an Anton Paar MCR-301 stress controlled rheometer with an environmental enclosure and Peltier temperature control. Temperature and frequency sweep measurements were performed using a 25 mm cone and plate geometry, with a 1° angle and 50 μm truncation gap. Creep measurements were performed using a 10 mm parallel plate geometry at a gap distance of 300 μm . Mineral oil was used as a solvent barrier to prevent sample dehydration. Creep

measurements at the specified temperatures were performed at two separate applied stresses in the linear range, each cycle consisting of 30 minutes under constant load followed by 30 minutes at zero load.

Results and Discussion

Effect of Protein Associating Domains on the Block Copolymer Self-Assembly Transition

The presence of associating domains within the protein midblock promotes microphase separation of PNIPAM domains to form aggregated nanostructures at a lower temperature and lower polymer concentration than in gels without associating domains in the protein midblock. Four different proteins were synthesized to examine the effect of midblock size and number of associative domains on gel nanostructure and mechanics: C_{80} , $C_{20}(PC_{20})_2$, $C_{10}(PC_{10})_4$,[&] and $C_{10}(PC_{10})_2$. The first three proteins have roughly similar molecular weight (~ 65 kDa) and vary only in the number of α -helical associating domains (0, 2, or 4), while the fourth was engineered to be roughly half the molecular weight of the other proteins (Table S1). Three batches of PNIPAM were prepared with molar masses of 6.4, 19.2, and 31.2 kDa (Figure S1), as summarized in Table 1. Nanostructure formation in 30% (w/v) hydrogels was studied via SANS at 5°C and 35°C (Figure 2), below and above the endblock transition temperature (T_t), which was determined by DSC to fall in the temperature range 19°C – 23°C (Table 2). Even below the T_t , many samples show a defined scattering peak indicating endblock segregation occurs despite solvation of the PNIPAM endblocks. The presence of a scattering peak at low temperature depends strongly on the molecular weight of the PNIPAM endblock. This peak appears at 5 °C in all samples for the largest endblock molar mass (31.2 kDa) and does not occur for any of the samples conjugated to the smallest endblock (6.4 kDa). The trend towards increasing segregation with increasing endblock molar mass is consistent with block copolymer self-assembly in good solvents for both blocks, where repulsive interactions result in $\phi_{ODT} \sim N^{-1}$ or $N^{-0.62}$ (where ϕ_{ODT} is the volume fraction at the order-disorder transition and N is the degree of polymerization) in the concentrated or semidilute regimes, respectively.⁵¹ In addition to the increase in total molecular weight, increasing PNIPAM endblock molecular weight moves the block copolymers closer to the symmetric composition (where $\phi_{PNIPAM} = \phi_{Protein}$), which is anticipated to decrease $(\chi N)_{ODT}$, the product of the Flory-Huggins interaction parameter and the degree of polymerization at the order-disorder transition.

For endblocks with intermediate molar mass (19.2 kDa), there is a clear difference in the strength of segregation—as determined by the intensity of the scattering peak—for a block copolymer with no associations in the protein midblock, compared with those that contain associative protein midblocks ($C_{20}(PC_{20})_2$, $C_{10}(PC_{10})_4$, and $C_{10}(PC_{10})_2$), regardless of the number of associating domains or size of the midblock. Endblock segregation is more favourable in these gels because the segregated state will have greater exchange entropy among the midblock associations, which is known to promote phase separation in solutions of associative polymers.^{32, 34, 35}

[&]This midblock protein has the same sequence as the one abbreviated as P4 in reference 32.

The detailed evolution of nanostructure upon heating was investigated in these hydrogels at 30% and 10% concentration, revealing that at high concentration a smooth transition from the weakly segregated state at low temperature occurs leading to well-defined structural features at high temperature (Figure 3a). For this study, a separate batch of materials with C₁₀(PC₁₀)₄ midblocks and 18.4 kDa endblocks was prepared. To study gels with specific triblock weight fractions (w_t), diblock and triblock copolymers synthesized with PNIPAM on one or both ends were blended together in controlled amounts. The detailed nanostructure evolution of 30% (w/v) gels at a triblock weight fraction of $w_t = 0.53$ reveals a moderate low temperature peak followed by a smooth evolution of structure with increasing temperature. The appearance of higher order features begins at approximately 17.5 °C, consistent with DSC data that indicate the thermal transition occurs over a broad temperature range, with onset and peak temperatures of approximately 16.8 °C and 24.6 °C, respectively. The temperature at which the structure forms is a strong function of the concentration of polymer. For a 10% gel with $w_t = 0.53$ (Figure 3b), SANS shows that the low q intensity increases above 15 °C, but a peak is not observed until 32.5 °C. DSC indicates that a thermal transition occurs with an onset and peak at 26.0 °C and 30.2 °C, respectively, much higher than in the 30% gels. The transition temperature for PNIPAM typically increases with decreasing concentration,⁵² consistent with the behaviour of these biohybrid materials.

T_t was determined by DSC for all midblock and endblock combinations at 30% (w/v) (Table 2), and was found to fall within a temperature range of 18–23 °C, but exhibited a strong concentration dependence (Table S2). For a given block copolymer, increasing concentration was seen to decrease T_t significantly, as expected. At 30%, all samples exhibited transition temperatures that were lower than that of their respective PNIPAM homopolymers, indicating that protein conjugation depressed T_t regardless of the protein midblock structure. Gels with the smallest endblock molecular weights exhibited the lowest T_t of all samples, while increasing endblock size typically raises the T_t closer to its homopolymer value. The T_t of PNIPAM can be affected by the solubility of any attached chemical modifications, and these effects are seen to decrease with increasing PNIPAM molecular weight.⁵³ This evidence suggests that increasing midblock protein fraction promotes endblock segregation and decreases T_t . This effect is consistent with the increase in T_t observed for fixed endblock molecular weight as the triblock fraction increases (Table S3); extrapolating this trend suggests that pure triblock copolymers have a higher T_t than pure diblock copolymers. For diblock copolymers, the PNIPAM block fraction is lower and the midblock protein should lower T_t more.

Effect of Molecular Design on Nanostructure Formation

At a 30% concentration and 35 °C, the scattering patterns for all gels reveal the formation of well-defined nanostructures due to PNIPAM aggregation (Figure 2), with length scales that are largely independent of the number of midblock associations but strongly dependent on the relative block fractions. The SANS patterns can be fit to the Percus-Yevick disordered hard sphere model, which is typically used to model scattering for block copolymer gels that self-assemble to form a network of interacting spherical micelles (Figures 4a, b and Table S2).^{32, 48} Both the hard sphere radii (R_{HS}) and core radii (r_o) increase with increasing endblock molecular weight, as is expected due to the increase in PNIPAM volume fraction.

For a given endblock molecular weight, the core radii show little dependence on the midblock sequence, suggesting that the aggregation number is relatively insensitive to midblock molecular weight and the density of associating groups. However, gels with $C_{10}(PC_{10})_2$ midblocks have consistently smaller hard sphere radii. This result indicates that the micellar coronae are thinner, which can be expected because the midblock molecular weight in these gels are roughly 60% that of the other three proteins. The trend that the micelle volume fractions (η) generally decrease for all samples with increasing endblock molar mass is consistent with what is expected from the increase in micellar radii observed across the samples. However, for a given endblock and midblock molar mass, η varies more significantly than might be expected based on the similarity of R_{HS} and r_o . In particular, gels with a high fraction of midblock associations ($C_{10}(PC_{10})_2$ and $C_{10}(PC_{10})_4$) were typically found to have the lowest micelle volume fractions. This result likely indicates that changes in the micelle packing geometry have occurred due to the midblock associations. The micelle volume fraction is also affected by differences in endblock conversion, which can change the overall micelle count in these gels since the total polymer concentration was fixed at 30% (w/v).

The effect of variations in endblock conjugation efficiency on the nanoscale structural parameters was examined by comparing blends of triblock and diblock copolymers with $C_{10}(PC_{10})_4$ midblocks at 35 °C, revealing that only small changes in micellar structure occur as the triblock weight fraction is varied from $w_t = 0.33$ to 0.53 (Figure 4c). For all three gel formulations, the core radii remain constant, suggesting that the aggregation number does not change significantly. However, as w_t increases, the number of PNIPAM endblocks increases, and the total number of micelles should increase to compensate. As w_t increases, the hard sphere radii decrease slightly and the volume fraction (η) increases, leading to a decrease in the interdomain spacing by ~10%. These results are consistent with both an increase in the total number of micelles and an increase in micellar attraction due to increased bridging.

Percus-Yevick model fits as a function of temperature provide a detailed picture of the evolution of structural length scales in the gels, demonstrating subtle structural changes that occur throughout the whole temperature range, even well above T_t (Figure 4d). For a 30% gel with $w_t = 0.53$, R_{HS} and η change by 3% and 2%, respectively, from 30–50 °C but r_o increases more significantly by 18% over the same temperature range. The growth of r_o despite strong exclusion of water by PNIPAM indicates that the micellar aggregation number is increasing as the temperature continues to rise above the T_t . This result implies that the total number of micelles decreases with increasing temperature if nearly 100% of PNIPAM chains are assumed to be in a micelle well above T_t . A decrease in micelle number is consistent with the slight decrease in η that is observed above approximately 30 °C. For the same gel prepared at a 10% concentration, a weak scattering peak occurs only at high temperature at approximately $q^* = 0.012 \text{ \AA}^{-1}$ (Figure 3b), corresponding to an average micellar radius of greater than 50 nm and consistent with a reduced number of micelles expected at lower gel concentration.

DPLS measurements confirmed that most samples remain optically isotropic throughout the temperature range from 5–50°C, suggesting that spherical nanodomains are observed in 30%

gels for most formulations, with the exception of the gel with $C_{10}(PC_{10})_4$ and 31.2 kDa endblocks. This anisotropic gel is weakly birefringent at low temperatures and exhibited a strong thermoresponsive birefringent signature with an onset temperature at approximately 16°C (Figure 5), suggesting the formation of a cylindrical phase at elevated temperatures. While the Percus-Yevick model is not appropriate for fitting data for anisotropic cylindrical phases, the primary peak position in the SANS data for $C_{10}(PC_{10})_4$ with 31.2 kDa endblocks is at approximately $q^* = 0.007 \text{ \AA}^{-1}$. This value indicates an average interdomain spacing of about 79 nm, significantly larger than the other gels in the study.

Access to this anisotropic phase is promoted by increasing either endblock molar mass to 31.2 kDa or overall gel concentration to 40% (w/v) (Table 3), as is generally expected since the effective PNIPAM nanodomain volume fraction becomes larger. However, it is notable that the birefringent phase boundary is depressed to lower concentrations when associations are present, as is seen in the 19.2 kDa series at 40% concentration. As concentration is reduced, only gels with the highest number of associating domains remain birefringent, as is the case for the $C_{10}(PC_{10})_4$ gels at 30% concentration. Surprisingly, a gel prepared with $C_{10}(PC_{10})_2$ midblocks and 31.2 kDa endblocks has the largest ϕ_{PNIPAM} of all the gels explored, yet it remains optically isotropic at 30% concentration throughout the measured temperature range. However, the association density (number of associations per midblock molecular weight) for $C_{10}(PC_{10})_4$ gels is approximately 17% larger than that of the $C_{10}(PC_{10})_2$ gels. These data indicate that midblock associations promote the formation of birefringent phases in the gels, shifting the anisotropic phase boundary to lower concentration and segregation strength (χN).

Maximizing Thermoresponsive Stiffening

Differences in thermoresponsive self-assembly between the block copolymers lead to changes in the modulus enhancement, illustrating important structure-property relationships for maximizing the responsive stiffening of these gels. Temperature-sweep oscillatory shear rheology (100 rad/s, $\gamma = 1\%$) was performed on each sample to investigate the effect of molecular design on the thermal evolution of the plateau modulus, revealing sigmoidal-like curves (Figure 6, S5, S6) with distinct differences observed in the low-temperature modulus (G'_{5C}), the peak modulus (G'_{peak}), and the temperature at which the peak modulus occurred (T^* , Figure 7). Increasing endblock molecular weight universally increased G'_{peak} and the reinforcement ratio (G'_{peak}/G'_{5C}), but simultaneously decreased T^* . Protein gels without reinforcing endblocks³² exhibit a shift in the crossover point but the plateau modulus is essentially constant over the temperature range 5–50 °C, suggesting that the non-monotonic behaviour of $G'(T)$ for certain samples originates from temperature-dependent changes in the micellar network. This decrease is also observed in gels with C_{80} midblocks and in other studies on purely synthetic PNIPAM triblock systems,⁵⁴ indicating that it is not a result of the protein associations.

The thermal evolution of nanostructure for the controlled w_t samples, as studied via SANS (Figure 4), suggests that the origin of this non-monotonic behaviour is a decrease in the number of micelles far above the T_t . Even above 30 °C, the micelle aggregation number continues to increase, as indicated by the monotonic increase in r_o with temperature. This

growth must be accompanied by a decrease in the number of micelles in the gel, which is confirmed by a slight decrease in the volume fraction of micelles (η) above approximately 30 °C. The decrease in T^* (from 43.6 °C to 40.8 °C) is accompanied by a decrease in the peak in η (from roughly 30.1 °C to 27.6 °C) as w_t changes from 0.53 to 0.33. Since the shear elastic modulus of a micellar gel is directly proportional to the concentration of micelles,³⁹ the structural changes observed via SANS are consistent with the existence of a peak in $G'(T)$.

For gels with roughly the same midblock molecular weight, the addition of associative domains in the midblock (from C_{80} to $C_{20}(PC_{20})_2$ to $C_{10}(PC_{10})_4$) leads to large increases in the gels' peak moduli due to the introduction of physical crosslinks in the micelle corona. The effect of these elastic contributions is an increase in the peak moduli by roughly a factor of 2.5 or 4 (for an additional 2 or 4 midblock associating domains, respectively), although this increase is slightly less in the case of the largest endblocks (Figure 7a). The addition of the midblock associations is predicted to increase the maximum number of elastically effective chains in each midblock by a factor of 3 and 5, respectively, if all chains were elastically effective.³² While these samples have endblock conversions that can differ by up to 20% due to changes in reactivity of the bioconjugation site as a function of midblock design, studies performed on samples with controlled triblock weight fractions suggested that the peak modulus was relatively independent of endblock conversion for large $C_{10}(PC_{10})_4$ over the range explored (Figure S7).

The gel exhibiting the greatest peak modulus and also the highest reinforcement ratio was the $C_{10}(PC_{10})_2$ midblock with the 31.2 kDa endblocks, which had an elastic modulus that transitioned from 9.1 kPa at 5 °C to a peak of 128.5 kPa at 36.4 °C, a 14.1 fold increase. When the midblock association density is the highest but midblock molecular weight is increased (from $C_{10}(PC_{10})_2$ to $C_{10}(PC_{10})_4$), gels exhibit nearly identical peak moduli for 6.4 kDa and 19.2 kDa endblocks, but when the endblock molar mass is increased further to 31.2 kDa, the smaller midblock has the largest modulus. This result is noteworthy because the $C_{10}(PC_{10})_4$ midblock forms an anisotropic phase, and in other triblock copolymer gel systems this has been observed to increase both gel modulus and toughness.⁵⁵ However, when comparing these two gels at fixed concentration, differences in the number of interacting micelles due to either the change in micelle morphology or a lower PNIPAM volume fraction may account for the difference in the peak moduli. Overall, PNIPAM volume fraction (Table 1) is not a strong determinant of peak modulus, indicating that optimizing this parameter alone does not maximize gel reinforcement. However, it was only when the density of corona associations was high in addition to the micellar core radii being large that both the peak modulus and the reinforcement ratio were maximized, illustrating that within the parameter space explored these variables were critical for achieving maximum responsive stiffening.

Effect of nanostructure formation on gel relaxation behaviour

Variations in endblock size and midblock associations enable control over the longest relaxation time of the gels as reinforcement is triggered by heating from 5–50 °C, providing the ability to resist plastic deformation due to creep. The longest stress relaxation time (τ_0)

was extracted from creep measurements as the ratio of the irrecoverable component of the compliance (J_o) divided by the steady state rate of compliance (J_{∞}) for each sample (Figure 8). For all gels, increasing endblock molar mass from 6.4 kDa to 19.2 kDa leads to a large improvement in the temperature dependence of τ_o . For example, for the $C_{20}(PC_{20})_2$ midblock the 19.2 kDa endblocks provide over an order of magnitude increase in relaxation time at 50 °C compared with the 6.4 kDa endblocks. For 19.2 kDa endblocks, gels with large midblocks (C_{80} , $C_{20}(PC_{20})_2$, and $C_{10}(PC_{10})_4$) exhibit monotonic increases in τ_o with increasing temperature, but increasing the PNIPAM block fraction further by increasing endblock size to 31.2 kDa or changing to the smaller midblock ($C_{10}(PC_{10})_2$) leads to non-monotonic temperature dependence of τ_o for all gels. As a result, at low temperatures gels with 31.2 kDa endblocks have the largest τ_o for a given midblock, while by 50 °C the gels with 19.2 kDa endblocks have a longer relaxation time. For example, the largest τ_o of all gels (18,500 s) was found for the C_{80} gel with 31.2 kDa endblocks at 20 °C, but as the temperature was increased further to 50 °C, τ_o decreased by nearly three-fold. While under certain conditions midblock associations led to slight increases the terminal relaxation times, this was not found to be the case in general, particularly for the largest endblock molecular weights.

The non-monotonic behaviour in relaxation time correlates with the decrease in peak temperature (T^*) seen in the plateau modulus for gels with the largest endblocks (Figure 7c), suggesting that the same changes in network structure at high temperatures can affect the relaxation dynamics. When the dominant relaxation mode is micelle ‘hopping’,³⁹ the energy barrier for relaxation is related to the cost of micellar deformation. This barrier is directly proportional to the elastic shear modulus and the micelle volume, and it will also be positively correlated with the micellar packing fraction, η . The elastic modulus decreases by the largest amount at 50 °C for gels with the 31.2 kDa endblocks (Figure S5), and SANS data suggest that η decreases as well (Figure 4c, S4). Measurements of τ_o on 30% (w/v) gels with $C_{10}(PC_{10})_4$ midblocks (18.4 kDa endblocks) confirmed that the relaxation times are independent of w_t (Figure 9), suggesting that ‘bridge-exchange’ by the destruction of active crosslinking chains is not the slowest relaxation mode in these samples.³⁹ In particular, in telechelic associative polymer solutions, it has been shown that the zero shear viscosity should monotonically increase with equilibrium fraction of bridging chains (ϕ_{eq}).⁴⁰ Although ϕ_{eq} cannot be measured directly for these gels, it is expected to increase with increasing w_t . That ‘bridge-exchange’ is not dominant is also supported by the fact that τ_o is non-monotonic for gels with the largest endblocks at the highest segregation strength (highest temperature), where the energetic cost for endblock pull-out should be greatest. At high temperature, relaxations among interacting coiled-coils occur significantly faster,³² suggesting that the deformation and diffusion of micelles dominate the gel’s relaxation behaviour.

Creep measurements below the T_t reveal the effect of microphase separation on the gels’ relaxation dynamics, indicating that even while swollen, segregated PNIPAM domains introduce a slow relaxation mechanism into the gels. This effect is observed as an increase in τ_o for all samples at 5 °C as endblock molecular weight increases. Measuring the elastic and viscous moduli as a function of temperature confirms this observation: block

copolymers with C₈₀ midblocks and 6.4 or 19.2 kDa endblocks behaved as viscoelastic liquids in the approximate range of 5–15 °C, but had $G' > G''$ at these low temperatures when the endblock molar mass was increased to 31.2 kDa (Figure S5). This transition corresponds to presence of a low temperature scattering peak in the SANS data, indicating that endblock segregation even without desolvation leads to the formation of a viscoelastic network. Frequency sweeps confirm that this network introduces a long relaxation time into the gels with C₈₀ midblocks (Figure 10a–c). The effect of microphase separated endblocks on gels with associating midblocks can be observed in the crossover point, which is observed to shift to lower frequency with increasing endblock molecular weight (Figure 10d–f). Therefore, even solvated PNIPAM aggregates clearly suppress the relaxation dynamics of the gels.

Conclusions

The influence of macromolecular design on the thermodynamics of nanostructure formation and the mechanical reinforcement of double physical network PNIPAM-protein-PNIPAM gels was explored in detail, allowing the formation of injectable materials that can rapidly change to exhibit moduli ranging up to 10⁵ Pa at 30% concentration and have terminal relaxation times on the order of 10⁴ seconds. Control of associating group density within the artificially engineered protein midblock and of the PNIPAM endblock molecular weight influences the gel nanostructure and is critical for maximizing reinforcement. Gels with the largest micellar cores and highest PNIPAM volume fractions exhibited the greatest reinforcement when corona crosslinking due to midblock associations was highest. Gel relaxation was slowed significantly for formulations that yielded larger micellar hard sphere radii, although non-monotonic behaviour was observed when the endblock fraction was largest. Control of both the associative protein network and micellar structure are critical for designing shear thinning gels that can be maximally reinforced at physiological temperatures for use in clinically relevant applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

- (a) Design of hybrid block copolymer containing two orthogonal associating functionalities.
(b) Schematic of thermoresponsive reinforcement by endblock self-assembly.

Fig. 2. Small angle neutron scattering of 30% (w/v) hydrogels in 100 mM phosphate buffer, pH = 7.6, as a function of endblock molar mass and midblock protein.

Fig. 3.

Fine resolution temperature sweep SANS for gels with $C_{10}(PC_{10})_4$ midblocks, and 18.4 kDa endblocks, and $w_t = 0.53$ at (a) 30% and (b) 10% (w/v) showing the thermal evolution of the nanostructure.

Fig. 4.

(a) Hard sphere radii (R_{HS}), core radii (r_o), and (b) micelle volume fraction (η) from Percus-Yevick fits to SANS data for 30% (w/v) hydrogels at 35°C. Fit parameters for $C_{10}(PC_{10})_4$ midblock with 18.4 kDa endblocks at 30% (w/v) (c) as a function of triblock weight fraction at 35°C and (d) as a function of temperature for $w_t = 0.53$.

Fig. 5. Depolarized light scattering of a 30% (w/v) hydrogel with a $\text{C}_{10}(\text{PC}_{10})_4$ midblock and 31.2 kDa endblocks as a function of temperature ($1^{\circ}\text{C}/\text{min}$).

Fig. 6. Characteristic temperature sweep linear oscillatory shear rheology of 30% (w/v) gels with $C_{10}(PC_{10})_4$ midblocks and 31.2 kDa endblocks (100 rad/s, $\gamma = 1\%$, 1°C/min).

Fig. 7.

(a) The peak elastic modulus (G'_{peak}) and 5 °C modulus ($G'_{5^{\circ}\text{C}}$), (b) reinforcement ratio ($G'_{\text{peak}}/G'_{5^{\circ}\text{C}}$), and (c) peak temperature (T^*) extracted from temperature-sweep rheology as a function of endblock molar mass and midblock nature.

Fig. 8.

Longest stress relaxation times computed from linear creep measurements as a function of endblock molar mass and midblock type.

Fig. 9. Longest stress relaxation time (τ_0) measurements from creep for 30% (w/v) gels with $C_{10}(PC_{10})_4$ midblocks and 18.4 kDa endblocks, as a function of triblock weight fraction (w_t).

Fig. 10.
5 °C frequency sweeps for 30% (w/v) gels with (a–c) C₈₀ midblocks and (d–f) C₂₀(PC₂₀)₂ midblocks, as a function of endblock molar mass.

Table 1

Properties of synthesized hybrid block copolymer hydrogels

	<i>Endblock Conversion</i>			<i>PNIPAM Volume Fraction^a</i>		
	6.4 kDa	19.2 kDa	31.2 kDa	6.4 kDa	19.2 kDa	31.2 kDa
Midblock (Molar Mass)						
C ₈₀ (67.1 kDa)	0.84 ±0.05	0.83 ±0.07	0.61 ±0.05	0.14 ±0.01	0.32 ±0.02	0.36 ±0.02
C ₂₀ (PC ₂₀) ₂ (61.1 kDa)	0.76 ±0.04	0.58 ±0.01	0.64 ±0.07	0.14 ±0.01	0.27 ±0.01	0.40 ±0.03
C ₁₀ (PC ₁₀) ₄ (63.3 kDa)	0.79 ±0.02	0.61 ±0.04	0.55 ±0.07	0.14 ±0.01	0.27 ±0.01	0.35 ±0.03
C ₁₀ (PC ₁₀) ₂ (37.0 kDa)	0.77 ±0.04	0.42 ±0.01	0.48 ±0.04	0.21 ±0.01	0.30 ±0.01	0.45 ±0.02

^aBased on bulk copolymer.

Table 2

Endblock thermal transition temperature (T_t , in °C) determined by DSC as a function of PNIPAM molar mass for 30% (w/v) hydrogels in 100 mM phosphate, pH = 7.6.

Midblock	6.4 kDa	19.2 kDa	31.2 kDa
C ₈₀	18.5	20.7	19.3
C ₂₀ (PC ₂₀) ₂	19.1	21.1	22.0
C ₁₀ (PC ₁₀) ₄	18.1	22.6	20.7
C ₁₀ (PC ₁₀) ₂	20.5	22.7	22.5
None ^a	24.4	25.9	25.6

^aData are for homopolymer solutions prepared at the effective ϕ_{PNIPAM} for 30% (w/v) gels: 5%, 9%, and 12% for gels with ~65 kDa midblocks and 6.4, 19.2, and 31.2 kDa endblocks, respectively.

Table 3

Onset of DPLS structural transition ($^{\circ}\text{C}$) for 40% (w/v) gels in 100 mM phosphate, pH = 7.6 as a function of endblock molar mass

Midblock	6.4 kDa	19.2 kDa	31.2 kDa
C ₈₀	--	--	12
C ₂₀ (PC ₂₀) ₂	--	11	13
C ₁₀ (PC ₁₀) ₄	--	12	11
C ₁₀ (PC ₁₀) ₂	--	13	12