Viscoelastic Behavior of Environmentally Sensitive Biomimetic Polymer Matrices

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ABSTRACT: By incorporating polysaccharide−peptide interactions into poly(ethylene glycol) hydrogels, we created a unique polymer matrix exhibiting characteristics imparted by both covalent and physical cross-links. Dynamic mechanical testing revealed that the properties of this system were sensitive to temperature changes and thermally reversible. At lower temperatures and higher frequencies, the elastic response was dominated by physical cross-links created by associations between heparin and heparin binding peptides; the matrices were stabilized by covalent cross-links at higher temperatures. Furthermore, the strength of the physical cross-links depended on the relative affinity between heparin and heparin binding peptides. This system could serve as a model to investigate how the presence of both physical and covalent cross-links influences the viscoelastic behavior of biomimetic polymeric materials.

Introduction

Both the directed assembly and the nanometer scale of biological polymers have fueled an interest in designing macromolecular materials with controlled architecture and environmental response. We have shown previously that purely physical interactions between heparin binding peptides and heparin can induce the assembly of environmentally responsive synthetic systems.[1] Here, we investigate whether the combination of physical and covalent bonds leads to dynamic mechanical properties that differ from those of purely physical networks or purely covalent networks. We speculate that these physical interactions play a larger role in extracellular matrix mechanics than previously considered;[2] protein affinity to polysaccharides, not just hydration due to fixed charge density within the polysaccharide, is a dominant contributor to the storage modulus.

Most biomimetic three-dimensional systems are based on modifications of gel-forming polymers. Some form covalent networks, such as functionalized poly(ethylene glycol) (PEG), while others form physical gels, such as alginate and poly(*N*-isopropylacrylamide).[3] Recent work with many of these systems has incorporated biological or synthetic polymers that mimic biology to create stimuli-responsive systems.[1,4−9] In general, these systems consist primarily of covalent networks engineered to degrade enzymatically or to recognize biological polymers or physical gels that exhibit thermo- or pH-reversible behavior. Many systems manipulate biopolymers to tailor mechanical properties, degradation rates, and other biological functions, but only limited examples of biomimetic materials have mechanical characteristics of both covalent networks and physical gels.[9−12]

An increasing body of literature is focusing on how interactions between biological molecules affect mechanical properties of tissues and how mechanical forces influence cellular behavior.[13−18] Within biological systems, the extracellular environment consists of a complex variety of proteins and polysaccharides that can physically or covalently associate with one another. For example, many types of extracellular matrices are rich in sulfated glycosaminoglycans, such as heparan sulfate or chondroitin sulfate, that have high affinities for various proteins. Enzymatic digestion of glycosaminoglycans within lung, mesenteric artery, and pericardium has resulted in significant changes to mechanical properties such as the elastic modulus and stiffness.[15,17,18] Furthermore, the incorporation of decorin into collagen fibers has resulted in increased tensile strength.[14] Therefore, the presence of polysaccharides and proteins within extracellular matrices not only provides biochemical signals to surrounding cells but also interacts with other biological polymers in a way that contributes to the overall bulk mechanical properties of various tissues.

Our system, illustrated by the schematic in Figure 1, consists of dynamic physical interactions and stabilizing covalent bonds. Multiarm PEG functionalized with vinyl sulfone moieties were covalently coupled to peptides containing polysaccharide binding domains (PBD). The addition of heparin facilitated the formation of weak gel-like structures in a manner similar to that of a completely physical matrix previously described.[1] When an enzymatically sensitive cross-linker peptide was added, the physical matrix was stabilized by the formation of covalent cross-links. We show herein that the physical properties provided by the heparin/PBD peptide interactions were environmentally responsive and dominant under certain conditions.

Materials and Methods

Materials. Eight-arm poly(ethylene glycol) (PEG) (average MW 20 000 g/mol; polydispersity 1.04) made by ethyoxylation of a hexaglycerine core was purchased from Nektar Therapeutics (Huntsville, AL). For PEG modification reactions, methanesulfonyl chloride, triethylamine, toluene, dichloromethane, β-mercaptoethanol, sodium hydroxide, tungstic acid, and thionyl chloride were purchased from Sigma-Aldrich (St. Louis, MO), and anhydrous diethyl ether was obtained from EM Science (Gibbstown, NJ). Regarding peptide synthesis, the amino acids, rink amide resin, HBTU, diisopropylethylamine, piperidine, and trifluoroacetic acid (TFA) were purchased from Advanced ChemTech (Louisville, KY). Pyridine, dimethylformamide, and dichloromethane were obtained from Mallinckrodt (Phillipsburg, NJ), Burdick and Jackson (Muskegon, MI), and Alfa Aesar (Ward Hill, MA), respectively. Ethanedithiol and diethyl ether were purchased from EM Science, and the triisopropylsilane and acetonitrile were from Sigma-Aldrich. For all conjugation reactions, rheological measurements, and hemolysis assays, heparin from porcine intestinal mucosa (average MW 18 000

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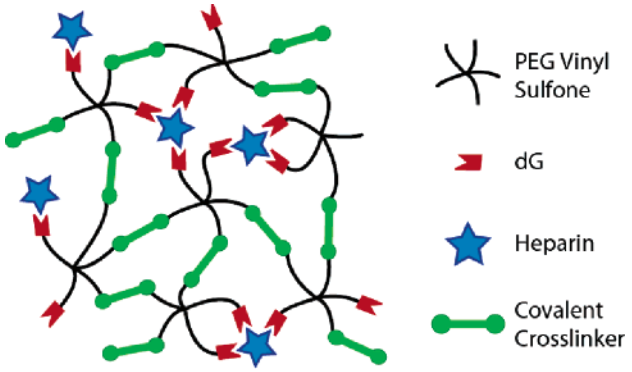

Figure 1. A schematic of a three-dimensional hydrogel incorporating covalent and physical cross-links. The peptides involved include dansyl-GKAFAKLAARLYRKAGC (dG) and GCRGDSGPQGIAGQGC (covalent cross-linker).

Table 1. Synthesized Peptides

designation	amino acid sequence
PBD	KAFAKLAARLYRKAGC
dG	dansyl-GKAFAKLAARLYRKAGC
W	WKAFAKLAARLYRKAGC
cross-linker or X	GCRGDSGPQGIAGQGC

g/mol) was obtained from Sigma-Aldrich, and Dulbecco's phosphate-buffered saline, pH 7.4 (PBS), was purchased from Invitrogen, Inc. (Carlsbad, CA). All of the water used throughout the study was treated through a Milli-Q Synthesis water purification system (Millipore, Billerica, MA; 18.2 MΩ·cm resistivity).

Synthesis and Characterization of Multiarm PEG Vinyl Sulfone. Terminal hydroxyl groups on the PEG polymers were functionalized with a vinyl sulfone moiety following the protocol described by Morpurgo et al.[19] Following synthesis, the multiarm PEG vinyl sulfone (PEGVS) was characterized using ^{1}H NMR spectra recorded in deuterated chloroform (Sigma-Aldrich) using a Gemini 300 MHz spectrometer (Varian, Inc., Palo Alto, CA). The degree of functionalization of the multiarm PEGVS was determined by comparing the average of the integrated areas of the peaks representing the three hydrogens of the vinyl group (δ 6.0−6.1, δ 6.3−6.4, and δ 6.8−6.9 ppm) with the integrated areas of the main PEG peak and associated sidebands (δ 3.3−3.9 ppm). The PEGVS was stored under argon at −20 °C.

Synthesis and Purification of Peptides. Standard Fmoc solid-phase chemistry was used for all peptide synthesis. The peptides listed in Table 1 were synthesized at a 0.7 mmol scale with rink amide resin using an Apex 396 peptide synthesizer (Advanced ChemTech). Fmoc-protected amino acids were coupled to the growing peptides through two successive additions of a 3 M excess amino acid, 6 M excess diisopropylethylamine, and 2.94 M excess HBTU. Fluorescent labeling of the dG peptide was performed by adding a 3 M excess of N-terminal dansyl glycine (Sigma-Aldrich) as described above for other amino acids. The peptides were cleaved from the resin using a cleavage cocktail consisting of 92.5% (v/v) TFA, 2.5% (v/v) Milli-Q water, 2.5% (v/v) triisopropylsilane, and 2.5% (v/v) ethanedithiol. Then, the peptides were precipitated in and washed three times with cold, anhydrous diethyl ether, centrifuged at 5000*g*, and dried in vacuo after decanting the ether.

Peptide purification was performed using reverse phase chromatography with an ÄKTA Explorer FPLC (Amersham Biosciences, Piscataway, NJ) equipped with a C18 column (Grace Vydac, Hesperia, CA; 22 mm internal diameter, 250 mm length, 10−15 μm particle size). After loading a peptide onto the column, a gradient consisting of 10−60% buffer B (acetonitrile with 0.1% TFA) in buffer A (Milli-Q water with 0.1% TFA) was run to elute the peptide. The collected peptides were lyophilized, and the mass of each peptide was confirmed with MALDI-TOF mass spectrometry on a Voyager-DE STR spectrometer (Applied Biosystems, Foster City, CA) using an α-cyano-4-hydroxycinnamic acid matrix (Sigma-Aldrich). All of the synthesized peptides were purified to greater than 95% purity as determined by reverse phase HPLC (data not shown).

Table 2. Compositions of Solutions and Gels[a]

designation	PEGVS	heparin (H)	cross-linker (X)	peptide
PEGVS-dG	●	○	○	dG
PEGVS-W	●	○	○	W
PEGVS-dG-H	●	●	○	dG
PEGVS-W-H	●	●	○	W
PEGVS-dG-X-H	●	●	●	dG
PEGVS-W-X-H	●	●	●	W
PEGVS-dG-X	●	○	●	dG
PEGVS-W-X	●	○	●	W
PEGVS-X	●	○	●	○
PEGVS-H	●	●	○	○

[a] The closed and open circles indicate the presence (●) and absence (○) of a particular chemical species. For the column "peptide", the designation of the heparin binding peptide used in the composition is given in lieu of a closed circle.

Conjugation of Peptides to PEGVS. Peptides were coupled to PEGVS through a Michael-type addition reaction involving the thiol of the cysteine residue within the peptide and the vinyl group on the PEGVS. To conjugate, on average, two heparin binding peptides to each PEG molecule, a 2-fold molar excess of peptide was combined with 1 equiv of PEG vinyl sulfone. To create a 10% (w/v) solution containing either 2:1 dG:PEGVS (composition PEGVS-dG) or 2:1 W:PEGVS (composition PEGVS-W), the PEGVS−peptide mixtures were solubilized in a 150 mM sodium phosphate buffer, pH 7.4, containing 5 mM EDTA. The solutions were incubated, protected from light, at room temperature for 2 h. Following the 2 h incubation, the conjugated PEG solutions were kept in an ice bath protected from light. The degree of peptide conjugation was observed by monitoring the disappearance of free thiols as indicated by an Ellman's assay.[20]

Heparin Affinity Liquid Chromatography. Affinity chromatography was performed on an ÄKTA Explorer FPLC equipped with a HiPrep 16/40 Heparin FF column (Amersham Biosciences). Buffer A consisted of 50 mM sodium phosphate, pH 7.4, and buffer B contained 50 mM sodium phosphate and 2 M sodium chloride, pH 7.4. Both buffers were degassed and filtered using a 0.2 μm filter (Nalge Nunc International, Rochester, NY). For all heparin affinity chromatography, the FPLC was run at 5 mL/min at room temperature. Before loading each peptide, the heparin column was equilibrated with buffer A. Purified peptides were solubilized in buffer A and loaded onto the heparin column by injecting 0.5 mL of a 1 mg/mL solution. Once each peptide was loaded onto the column, a gradient of 0−100% buffer B in buffer A was carried out over 20 column volumes. The sodium chloride concentration required to elute each peptide was determined as the gradient value at the time when the absorbance at 280 nm reached a maximum.

Dynamic Mechanical Properties. The viscoelastic mechanical properties of the PEG-*co*-peptide/heparin/cross-linker materials were measured with an AR1000 rheometer (TA Instruments, New Castle, DE) using an acrylic cone and plate geometry with a 20 mm diameter, 50 μm truncation and 1°59′ cone angle. The temperature of the rheometer surface was controlled with a built-in Peltier system. All described compositions contained a final volume of 100 μL with a polymeric content (as determined by the amount of PEGVS-*co*-peptide and heparin within the material) of 10% (w/v) and were formed on the rheometer surface maintained at 20 °C. Table 2 shows a list of the designations and general makeup of each composition; a more detailed description of each composition can be found in the Supporting Information. Briefly, compositions PEGVS-dG-H and PEGVS-W-H were created by combining PEGVS-dG or PEGVS−W, respectively, and heparin in a 9:1 molar ratio of peptide to heparin. Then, a 1.5 molar excess of difunctional cross-linker peptide, relative to PEGVS, was added to in order to introduce stabilizing covalent bonds within the materials. Materials containing PEGVS-dG, heparin (H), and cross-linker (X) were designated as composition PEGVS-dG-X-H; materials containing PEGVS-W, heparin, and cross-linker were designated as composi-

tion PEGVS-W-X-H. In all compositions, 150 mM sodium phosphate, pH 7.4, with 5 mM EDTA was used as the buffer, and three mixtures of all compositions were formed and characterized. Controls consisted of PEGVS-dG and PEGVS-W materials formed with cross-linker peptide but without heparin (compositions PEGVS-dG-X and PEGVS-W-X, respectively) as well as PEGVS materials lacking heparin binding peptides but containing either cross-linker peptide or heparin (compositions PEGVS-X and PEGVS-H, respectively).

As soon as all of the material components had been added and mixed, the rheometer geometry was lowered until it was 50 μm above the plate, and the outer diameter of the geometry was covered in mineral oil to prevent evaporation. Initially, one material composition of each type was subjected to a preliminary stress sweep to ensure that the viscoelastic response remained in the linear range. The stress sweep was performed by applying 0.1−1000 Pa stresses at 10 rad/s at 20 °C. All rheological data were collected using the following steps. Immediately after lowering the geometry, a time sweep was conducted by applying a 0.5 Pa oscillatory stress for 2 h at 5, 10, 15, 20, 25, 30, 35, and 40 rad/s at 20 °C. Next, three frequency sweeps were performed by applying a 0.5 Pa oscillatory stress throughout a 0.1−40 rad/s frequency range at 20, 4, and 37 °C. The frequency sweeps were followed by temperature sweeps during which viscoelastic properties were measured by applying a 0.5 Pa oscillatory stress at 10 rad/s from 4 to 45 °C and from 45 to 4 °C. At each target temperature within the 4−45 °C range, the temperature was allowed to equilibrate for 15 s before a measurement was recorded. For all rheological studies, t-tests and ANOVA were performed ($\alpha = 0.05$) to determine statistical significance.

Results and Discussion

PEGVS Synthesis. ^{1}H NMR characterization of the PEGVS showed distinct peaks centered around 6.07, 6.35, and 6.81 ppm, indicating the presence of vinyl protons (data not shown). The degree of functionalization was calculated by comparing the ratio of the average integral of these three peaks with the main PEG peak around 3.6 ppm. This analysis showed that, on average, 5.0 of the 8 arms were modified with a vinyl sulfone residue. Although the degree of substitution was only 62.5%, the 5-arm functional PEGVS polymer provided a unique backbone on which to couple several types of peptides. The degree of functionalization was confirmed by conjugating various ratios of cysteine to the PEGVS and evaluating the presence of free thiols using Ellman's assay as well as by using various ratios of dithiothreitol to form 5% (w/v) gels (data not shown).

Heparin Affinity Liquid Chromatography. Heparin-binding peptides were synthesized with different N-terminal domains in an attempt to understand how the presence of two different fluorescent labels (dansyl and tryptophan) affected the peptides ability to bind to a heparin column relative to an unmodified peptide derived from the heparin binding domain from antithrombin III.[21] Figure 2 shows the relative heparin binding ability of PBD, W, and dG. As indicated by the concentration of sodium chloride required for elution from a heparin column, the addition of a hydrophobic moiety to the N-terminus of PBD resulted in a dramatic increase in heparin affinity. For example, in contrast to the 740 mM NaCl needed to elute dG, PBD eluted at a concentration of 380 mM NaCl or nearly half of that of dG. Although not as strong as the dG affinity to heparin, W required about 20% more sodium chloride (460 mM) before eluting compared with the elution profile of PBD. In addition to increasing heparin affinity, the N-terminal dansyl glycine and tryptophan residues increased solubility of the peptide in physiologically relevant buffers (data not shown). These improved solubilities and superior heparin binding characteristics were two reasons for selecting dG and W as the peptides for use in the creation of physical cross-links in various gel compositions.

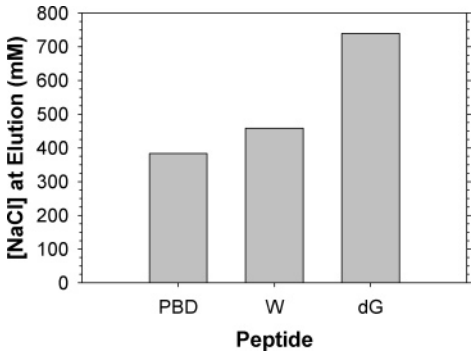

Figure 2. Relative heparin affinities of three peptides derived from antithrombin III.

Previous work has shown that peptides with similar primary structures bind to heparin with very different affinities.[1,21] As shown in Figure 2, data describing relative heparin affinities of PBD, W, and dG further demonstrated that small changes in the molecular structure of a peptide, including the addition of various fluorescent labels, can greatly affect peptide properties. Regarding the relative increase in heparin binding for the W and dG peptides compared with PBD, one hypothesis remains that the hydrophobic core of the fluorophore was shielded from water by the pendent groups of the more hydrophilic functional groups of lysine and arginine amino acids prevalent within the cationic peptides. This shielding may have led to changes in peptide secondary structure resulting in favorable thermodynamic properties influencing solubility and affinity for anionic heparin. Some preliminary experiments using circular dichroism to study these heparin binding peptides have suggested that W assumes a more α-helical structure relative to PBD in water and in the presence of heparin (data not shown).

Dynamic Mechanical Properties. Figure 3 displays the storage modulus, G', and the loss modulus, G'', as a function of curing time for gels of composition PEGVS-dG-X-H, PEGVS-W-X-H, PEGVS-dG-X, and PEGVS-X. Although measured at 40 rad/s, the relative moduli magnitudes between gel compositions as well as associated trends remained similar even when the stress was applied at 5 rad/s (data not shown). Compositions PEGVS-dG-H and PEGVS-W-H showed frequency-dependent, but extremely weak, gel-like behavior with trends similar to physical matrices previously described (data not shown).[1] Composition PEGVS-H did not result in the formation of a gel but rather remained as a solution throughout the duration of the rheological experiments (data not shown). When examining Figure 3a, the time to gelation, t_{gel}, of gels of composition PEGVS-dG-X (30.0 ± 4.5 min) was significantly longer ($p < 0.05$) than the t_{gel} of the heparin-containing composition PEGVS-dG-X-H (7.3 ± 0.6 min). Both G' and G'' of the gels containing dG and heparin were significantly higher than the moduli of similar gels without heparin; after 2 h, G' of composition PEGVS-dG-X-H (2760 ± 320 Pa) was over 10 times larger than the 260 ± 60 Pa magnitude of G' recorded for composition PEGVS-dG-X. As the association of dG and heparin is rapid, the mechanical properties of the compositions were likely affected by the formation of larger macromolecular chains coordinated by physical bonds. The resulting long chain intermolecular interactions stabilized the materials, which were further stabilized by chemical cross-links introduced by the cross-linker. Consequently, the influence of the heparin-related

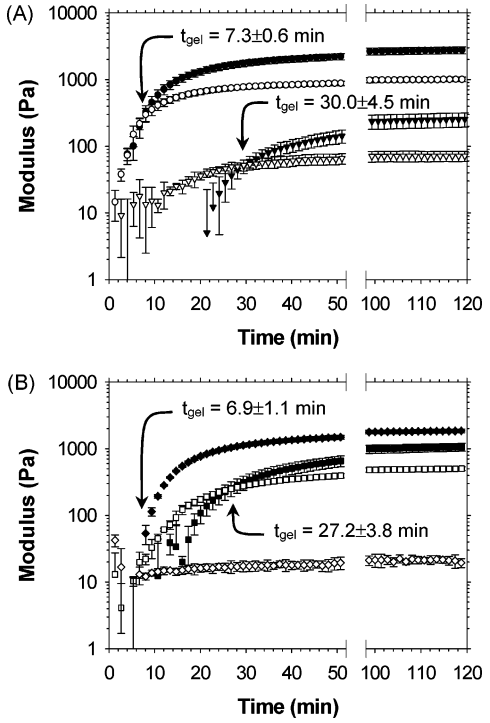

Figure 3. A plot showing the viscoelastic properties of various gels, measured at 40 rad/s, as a function of time ($n = 3$; ±SD). Panel A depicts G' and G'' for gels of composition PEGVS-dG-X-H (● and ○, respectively) and PEGVS-dG-X (▼ and ▽, respectively). Panel B depicts G' and G'' for gels of composition PEGVS-W-X-H (■ and □, respectively) and PEGVS-X (◆ and ◇, respectively). The time t_{gel} represents the gelation time at which the increasing magnitude of G' is equal to that of G''.

physical coordination resulted in decreased t_{gel} and larger moduli.

When comparing the mechanical response of gels of composition PEGVS-dG-X-H with the data reported in Figure 3b, we observed that the t_{gel} of composition PEGVS-dG-X-H was significantly lower than that for similar gels of composition PEGVS-W-X-H (27.2 ± 3.8 min) in which dG was replaced with W. In addition, the magnitude of G' for composition PEGVS-W-X-H (1060 ± 140 Pa) was significantly lower than that for gels of composition PEGVS-dG-X-H but statistically higher than that for gels of composition PEGVS-dG-X. This result indicated that interactions between heparin and heparin binding peptides influenced the mechanical properties of the gel-like material in a manner dependent on the relative affinity of various peptides for heparin. While the net cationic charge of the two peptides remained the same, W had a lower affinity for heparin relative to dG. Therefore, although still present, the contribution from heparin−peptide interactions to the mechanical response of the composition was not as prominent for gels containing the lower affinity peptide.

Relative to gels of composition PEGVS-dG-X-H, the t_{gel} of PEG gels of composition PEGVS-X (6.9 ± 1.1 min), which represented a formulation containing the theoretical maximum number of covalent cross-links between PEGVS molecules, was not significantly different. After 2 h, G' of PEGVS-X gels (1860 ± 120 Pa) was statistically lower than that recorded for PEGVS-dG-X-H. It is important to note that the lower modulus resulting from composition PEGVS-X might be due, at least in part, to an effective covalent cross-link density lower than that theoretically calculated. This lower cross-link density could have resulted either from cross-linker peptide covalently binding two vinyl sulfone residues on the same PEGVS molecule rather than linking two distinct PEGVS molecules together or from inefficient covalent cross-linking. Consequently, gels of composition PEGVS-X would have experienced a decrease in the magnitude of G'. It is likely that the elastic response of all of the compositions were affected by inefficient cross-linking as well as polydispersities introduced by PEGVS and heparin. Regardless, the magnitude of the moduli for composition PEGVS-dG-X-H demonstrated that physical interactions can greatly affect the transient and equilibrated mechanical response of hydrogels.

Although an in-depth analysis regarding the initial kinetics of the PEGVS-dG-X-H gels remains better suited for future work, it appeared that strong affinities between heparin and dG influenced the initial shape and rise of both G' and G'' relative to the time-dependent viscoelastic profile of PEGVS-dG-X. The profiles of $G'(t)$ and $G''(t)$ for PEGVS-dG-X gels appeared to follow trends observed for soft, self-assembled materials, such as collagen, in that $G''(t)$ displayed a sigmoidal-like rise and $G'(t)$ rose rapidly after an initial delay (Figure 3).[22] This observation paralleled typical sol−gel viscoelastic behavior, and future analysis will attempt to determine whether the gelation kinetics of this material as well as other compositions examined herein follow known power law kinetics seen with other materials.[22] In contrast to the results of PEGVS-dG-X, both $G''(t)$ and $G'(t)$ for PEGVS-dG-X-H gels initially rose very rapidly and seemingly at similar rates. This behavior appeared limited to gels containing peptides with high heparin affinity because the time-dependent viscoelastic profile of PEGVS-W-X-H at 20 °C was more analogous to that of PEGVS-dG-X than PEGVS-dG-X-H. At temperatures lower than 20 °C, the behavior of PEGVS-W-X-H might more closely resemble that of PEGVS-dG-X-H because, as seen below, the novel compositions described herein were extremely sensitive to temperature. In previous studies, we have shown that the coordination of physical bonds between PEGVS-dG and heparin occurred essentially immediately upon mixing and were too fast to be measured using rheology.[1] Therefore, the rapid and steep rises of G' and G'' of PEGVS-dG-X-H likely were influenced by the rapid association between heparin and heparin binding peptides as well as by effects due to mixing. Overall, viscoelastic parameters of PEGVS-dG-X-H and PEGVS-W-X-H would largely depend on factors such as temperature, frequency, concentrations, ionic strength, and pH. However, care must be taken when analyzing the transient viscoelastic response of PEGVS-dG-X-H and PEGVS-W-X-H since the kinetic mechanisms of gel assembly involved many complex parameters including polydispersities of the PEGVS and heparin, viscous effects due to the presence of heparin, and reaction and diffusion events between both heparin and PEGVS-dG and PEGVS-dG and covalent cross-linker.

After curing, gels of composition PEGVS-dG-X-H, PEGVS-W-X-H, PEGVS-dG-X, and PEGVS-X were subjected to a frequency sweep at 20 °C. As seen in Figure 4a, the elastic response of gels of composition PEGVS-dG-X (on average 260 ± 100 Pa) was indicative of a covalent gel network and remained frequency independent throughout the tested frequency range. When heparin was added, the magnitude of G', as seen for composition PEGVS-dG-X-H, became dependent on the frequency of the applied stress. At 0.1 rad/s, G' was 480 ± 160 Pa and rose in a linear fashion (on a log−log plot) to 2810 ± 320 Pa at 40 rad/s. Throughout the frequency sweep, the magnitudes of G' for gels of composition PEGVS-dG-X were not statistically different from each other. However, a comparison of G' at each frequency reveals that the magnitude of G' for gels of composition PEGVS-dG-X-H was significantly

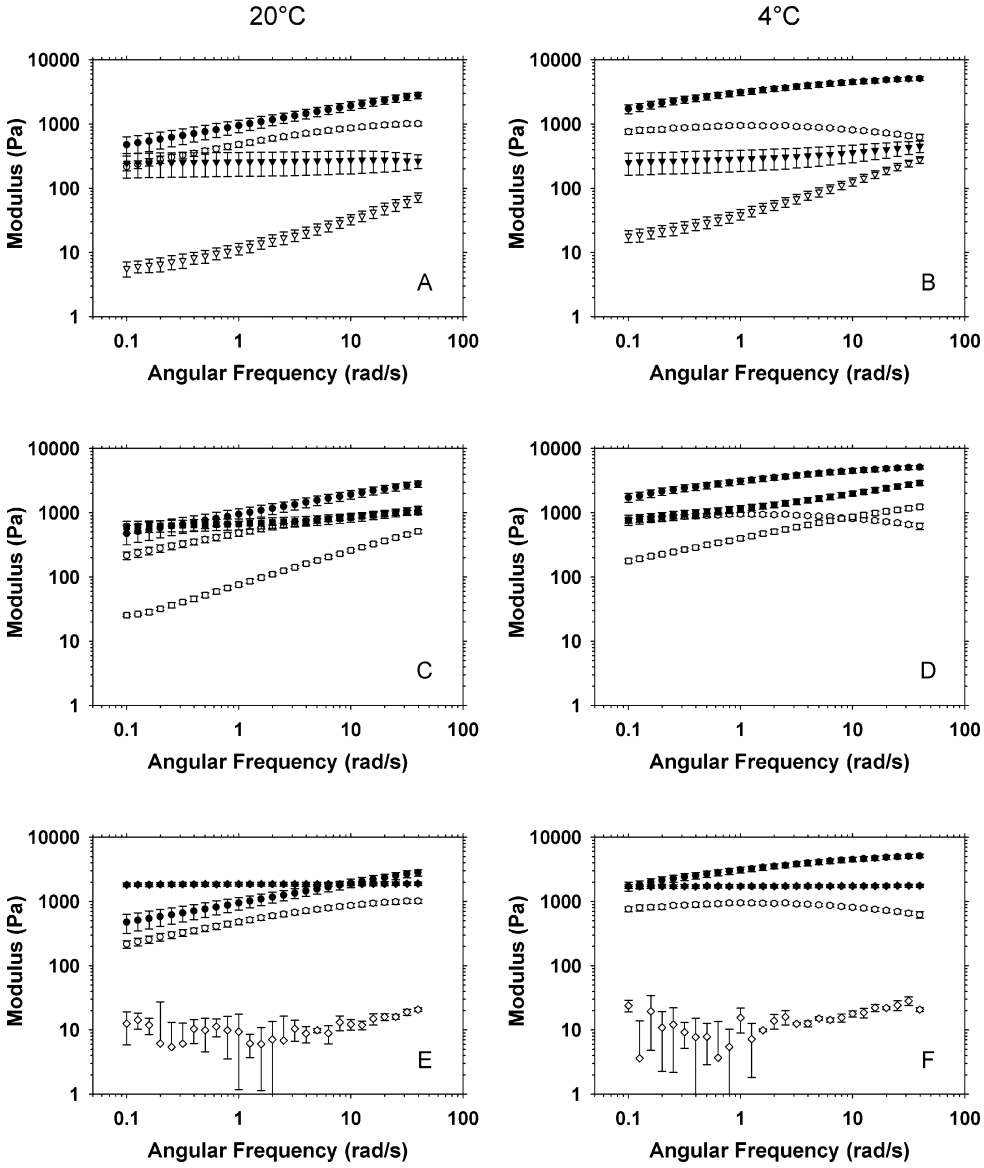

Figure 4. A plot of the viscoelastic properties of various gels as a function of frequency ($n = 3$; ±SD). G' and G'' for gels of composition PEGVS-dG-X-H (● and ○, respectively) and PEGVS-dG-X (▼ and ▽, respectively) are compared when measured at 20 °C (panel A) and at 4 °C (panel B). G' and G'' for gels of composition PEGVS-dG-X-H (● and ○, respectively) and PEGVS-W-X-H (■ and □, respectively) are compared when measured at 20 °C (panel C) and at 4 °C (panel D). G' and G'' for gels of composition PEGVS-dG-X-H (● and ○, respectively) and PEGVS-X (◆ and ◇, respectively) are compared when measured at 20 °C (panel E) and at 4 °C (panel F).

higher ($p < 0.05$) than that for composition PEGVS-dG-X for all frequencies above 0.1 rad/s. In addition, the viscoelastic response of composition PEGVS-dG-X-H indicated that the materials remained gel-like throughout the entire tested frequency range unlike purely physically associated matrices described in previous work.[1] Thus, the presence of covalent bonds between molecules of PEGVS-dG was able to stabilize the gels of composition PEGVS-dG-X-H without eliminating the frequency-dependent characteristics of the mechanical response due to physical interactions resulting from transient ionic and hydrogen bonds. The effect of physical bonds on dynamic mechanical properties within the system were magnified at 4 °C (Figure 4b). Although G' of PEGVS-dG-X remained independent of frequency and similar in magnitude to values measured at 20 °C, G' of PEGVS-dG-X-H at 4 °C was statistically higher than that measured at 20 °C at each frequency. For example, G' of PEGVS-dG-X-H at 4 °C increased from 1720 ± 270 Pa at 0.1 rad/s to 5130 ± 350 Pa at 40 rad/s. Thus, the viscoelastic nature of this system was significantly influenced by the frequency- and temperature-dependent physical associations formed between heparin and heparin binding peptides.

Figure 4c,d further demonstrate how both physical and covalent bonds contributed to the overall dynamic mechanical properties of the described system. When dG was replaced with the weaker heparin binding W peptide in composition PEGVS-W-X-H, the storage modulus at 20 °C still exhibited a frequency-dependent response by increasing in magnitude from 610 ± 130 Pa at 0.1 rad/s to 1110 ± 150 Pa at 40 rad/s (Figure 4c). At low frequencies, the elastic modulus of both composition PEGVS-dG-X-H and composition PEGVS-W-X-H were statistically similar. In theory, both of these compositions had the same covalent cross-link density and only differed in the individual peptide affinity for heparin. We have shown previously that the dynamic mechanical properties of physical networks based upon interactions between heparin and dG bound to PEGVS resulted in a frequency-dependent profile indicative of a high molecular weight polymer melt.[1] At low frequencies, that system was influenced primarily by a viscous response. For the system described herein, the viscoelastic properties of

gels consisting of either PEGVS-dG-X-H or PEGVS-W-X-H appeared to exhibit similar characteristics. When the frequency of the applied stress was too low to capture the transient binding between heparin and heparin binding peptides, the resulting mechanical properties were dominated by the elastic behavior influenced by the presence of covalent bonds. At 20 °C, once the frequency of the applied stress reached 1.259 rad/s, the G' of composition PEGVS-dG-X-H became significantly larger than that of composition PEGVS-W-X−H ($p < 0.05$). By applying a stress at increased frequencies, the transient physical bonds could have more heavily influenced the viscoelastic response and allowed G' of composition PEGVS-dG-X-H to reflect the presence of not only covalent interactions within the network but also stronger physical associations relative to those in composition PEGVS-W-X-H. When the temperature dropped to 4 °C, G' of PEGVS-dG-X-H was significantly larger than that of composition PEGVS-W-X-H at all frequencies (Figure 4d). This trend correlated well with the relative heparin affinities of the dG and W peptides and suggested that although the elastic response of both compositions appeared to increase at a similar rate as a function of angular frequency, the system remained sensitive to the kinetics of peptide/polysaccharide interactions even at low temperatures.

The data presented in Figure 4e,f are similar to that in Figure 4a,b in that the frequency-dependent viscoelastic response of composition PEGVS-dG-X-H is compared with the frequency-independent elastic response of a gel with a different composition. In this case, Figure 4e,f displays the mechanical properties of composition PEGVS-X. At 20 °C, G' of composition PEGVS-X remained unaffected by frequency and has an average magnitude of 1860 ± 130 Pa throughout the tested frequency range (Figure 4e). For frequencies lower than or equal to 3.981 rad/s, G' of composition PEGVS-X was significantly larger ($p < 0.05$) than G' of composition PEGVS-dG-X-H. Between 3.981 and 19.98 rad/s, the elastic moduli of both compositions were similar at each frequency, and G' of composition PEGVS-dG-X-H was statistically higher than G' of composition PEGVS-X at frequencies equal to or above 19.98 rad/s. At 4 °C (Figure 4f), G' of PEGVS-X had an average value of 1730 ± 110 Pa, which was not different than that measured at 20 °C. Since the lower temperature effectively allowed the physical bonds of composition PEGVS-dG-X-H to play a more dominant role in bulk viscoelastic properties, the magnitude of G' of PEGVS-dG-X-H was significantly higher than that of composition PEGVS-X for all frequencies at or above 0.2 rad/s. Although composition PEGVS-dG-X served as a better control for evaluating how the presence of physical cross-links affected a covalently cross-linked network, composition PEGVS-X was used to compare gels containing both covalent and physical interactions with gels consisting purely of a strong, covalently bound material. Care must be taken when comparing the properties of composition PEGVS-dG-X-H with those of composition PEGVS-X due to differences in PEGVS and cross-linker content that would influence not only equilibrium properties but also the rate of cross-linking during gel curing. Even while acknowledging the limitations of a direct comparison, it was impressive that there existed a frequency range in which a combination of covalent and physical cross-links resulted in a larger G' compared with a similar gel consisting only of covalent cross-links. Taken together, the observations made from Figure 4 demonstrated that the presence of physical interactions significantly altered the mechanical properties of three-dimensional gels.

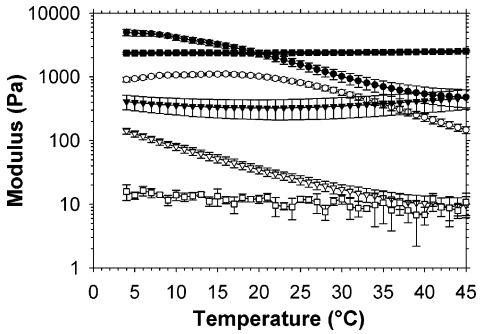

Figure 5. A plot of the viscoelastic properties of various gels as a function of temperature ($n = 3$; ±SD). All of the temperature sweeps were measured using an angular frequency of 10 rad/s. G' and G'' are shown for gels of composition PEGVS-dG-X-H (● and ○, respectively), PEGVS-dG-X (▼ and ▽, respectively), and PEGVS-X (■ and □, respectively).

In previous work, we have shown that the viscoelastic behavior of physical matrices consisting of associations between heparin and heparin binding peptides depends on several parameters including frequency of the applied stress and temperature.[1] In this work, we wanted to examine whether physical matrices stabilized by covalent cross-links would continue to respond to temperature variations. As seen in Figure 5, G' of gels of composition PEGVS-dG-X was relatively independent of temperature. In fact, G' at 4 °C (410 ± 100 Pa) was not significantly different ($p > 0.05$) than G' at 45 °C (470 ± 160 Pa). Similarly, G' of gels of composition PEGVS-X was not influenced by temperature and averaged 2400 ± 90 Pa throughout the temperature sweep. The temperature independence of these data showed that gels without heparin behaved in a manner consistent with other purely covalently cross-linked networks.

In contrast, the viscoelastic properties of gels of composition PEGVS-dG-X-H displayed remarkable temperature dependence as well as little to no hysteresis when the temperature was cooled from 45 °C down to 4 °C (data not shown). G' at 4 °C (5000 ± 590 Pa) was significantly higher ($p < 0.05$) than G' at 45 °C (480 ± 150 Pa). Since the interaction between anionic heparin and cationic dG resulted primarily from physical electrostatic associations, the properties imparted by these interactions were transient and dynamic. At low temperatures, the gel components likely experienced lower diffusion coefficients as well as lower kinetic rate constants, resulting in an increased time of contact between dG and heparin. When the energy of the system increased, the number of physical bonds at any given time decreased until the interaction between heparin and dG no longer made a significant contribution to the elastic properties of the gel. G' of gels of composition PEGVS-dG-X-H and PEGVS-dG-X between 36 and 45 °C were not statistically different at each temperature, suggesting that above 35 °C covalent interactions contributed wholly to the elastic properties of the gels. This temperature-dependent gel weakening exhibited the same trend seen for previously described physical matrices.[1] However, unlike the moduli of the completely physically associated materials, G' remained higher than G'' throughout the entire tested temperature range for all of the gel compositions reported herein.

Also interesting was the observation that the average magnitude of G' for heparin-containing gels (composition PEGVS-dG-X-H) at 4 °C was 2.1 times larger than that for the covalently cross-linked gels of composition PEGVS-X and remained higher at each temperature through 18 °C ($p < 0.05$). At 45 °C, the value of G' for gels of composition PEGVS-dG-X-H was lower

than that of composition PEGVS-X by a factor of 5.3. Although weaker by 2 orders of magnitude at 37 °C, the elastic properties of these 10% gels at 4 °C were only about 2.5-fold lower than those published for 15% and 40% covalent PEG-based hydrogels tested at 37 °C.[23,24] By making this comparison, it is not suggested that the materials described herein could provide superior results to those referenced; rather, from the perspective of better understanding the role of physical cross-links within extracellular matrices, the elastic moduli recorded for gels of composition PEGVS-dG-X-H at low temperatures indicated that physical interactions can significantly influence and strengthen the mechanical properties of hydrogels. Obviously, one major limitation of the described system remains the retention of physical cross-links at physiologically relevant temperatures. Even though the magnitude of the viscous response of gels of composition PEGVS-dG-X-H at 37 °C was over 30 times larger than that of gels of composition PEGVS-dG-X (310 ± 40 and 10 ± 3 Pa, respectively), it would be useful to have a stronger elastic response resulting from physical cross-links to be present within the gels at higher temperatures in order to study the effect of such cross-links on cellular behavior or evaluate this material system as a vehicle for drug or gene delivery. It is important to remember that the viscous response of gels of composition PEGVS-dG-X-H remained much higher than G'' for gels of composition PEGVS-dG-X throughout the tested temperature range. Thus, the mechanical properties of gels with physical bonds, although dominated by an elastic response, also displayed a large dependence on the viscous response, indicating that the heparin/peptide interactions continued to influence the viscoelastic behavior of the gels. Future experiments will investigate whether this influence is sufficient to affect the response of the gels in various biological applications.

Conclusion

The gel system described herein represents a unique material that incorporates properties of both physically and covalently cross-linked hydrogels and that demonstrates that physical interactions can play a dominant role in the determination of mechanical properties within a polymer matrix. Because of the presence of physical associations between heparin and dG and unlike most covalent systems, the mechanical properties of these gels were sensitive to temperature. This temperature dependence could be tailored with various anionic polysaccharides or peptides with different heparin affinities to control dynamic mechanical properties, influence swelling behavior, and/or provide a mechanism for releasing or recognizing molecules based on local temperature. The properties of this system could also be modified by increasing the number of functional PEG arms or by using other multivalent polymers, such as dextran. In addition to temperature changes, the physical interactions within the gels would likely be influenced by pH and ionic strength. Consequently, this system could be useful as a model to better understand how physical and covalent cross-links interact to influence the behavior of biopolymeric materials.

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Supporting Information Available: A detailed description of the compositions of solutions and gels and frequency sweep data for various gels measured at 37 °C. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Seal, B. L.; Panitch, A. *Biomacromolecules* **2003**, *4*, 1572−1582.
(2) Sun, D. D.; Guo, X. E.; Likhitpanichkul, M.; Lai, W. M.; Mow, V. C. *J. Biomech. Eng.* **2004**, *126*, 6−16.
(3) Seal, B. L.; Otero, T. C.; Panitch, A. *Mater. Sci. Eng. R* **2001**, *34*, 147−230.
(4) Panitch, A.; Yamaoka, T.; Fournier, M. J.; Mason, T. L.; Tirrell, D. A. *Macromolecules* **1999**, *32*, 1701−1703.
(5) Petka, W. A.; Harden, J. L.; McGrath, K. P.; Wirtz, D.; Tirrell, D. A. *Science* **1998**, *281*, 389−392.
(6) Yamaguchi, N.; Chae, B. S.; Zhang, L.; Kiick, K. L.; Furst, E. M. *Biomacromolecules* **2005**, *6*, 1931−1940.
(7) Lutolf, M. P.; Raeber, G. P.; Zisch, A. H.; Tirelli, N.; Hubbell, J. A. *Adv. Mater.* **2003**, *15*, 888−892.
(8) Wang, C.; Kopecek, J.; Stewart, R. J. *Biomacromolecules* **2001**, *2*, 912−920.
(9) Kim, S.; Healy, K. E. *Biomacromolecules* **2003**, *4*, 1214−1223.
(10) Cellesi, F.; Tirelli, N.; Hubbell, J. A. *Biomaterials* **2004**, *25*, 5115−5124.
(11) Miyata, T.; Asami, N.; Uragami, T. *Nature (London)* **1999**, *399*, 766−769.
(12) Cohn, D.; Sosnik, A.; Garty, S. *Biomacromolecules* **2005**, *6*, 1168−1175.
(13) Tan, J. L.; Tien, J.; Pirone, D. M.; Gray, D. S.; Bhadriraju, K.; Chen, C. S. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 1484−1489.
(14) Pins, G. D.; Christiansen, D. L.; Patel, R.; Silver, F. H. *Biophys. J.* **1997**, *73*, 2164−2172.
(15) Gandley, R. E.; McLaughlin, M. K.; Koob, T. J.; Little, S. A.; McGuffee, L. J. *Am. J. Physiol.* **1997**, *42*, H952−H960.
(16) Redaelli, A.; Vesentini, S.; Soncini, M.; Vena, P.; Mantero, S.; Montevecchi, F. M. *J. Biomech.* **2003**, *36*, 1555−1569.
(17) Mavrilas, D.; Sinouris, E. A.; Vynios, D. H.; Papageorgakopoulou, N. *J. Biomech.* **2005**, *38*, 761−768.
(18) Al Jamal, R.; Roughley, P. J.; Ludwig, M. S. *Am. J. Physiol.* **2001**, *280*, L306-L315.
(19) Morpurgo, M.; Veronese, F. M.; Kachensky, D.; Harris, J. M. *Bioconjugate Chem.* **1996**, *7*, 363−368.
(20) Ellman, G. L.; Courtney, K. D.; Andres, V.; Featherstone, R. M. *Biochem. Pharmacol.* **1961**, *7*, 88.
(21) Tyler-Cross, R.; Sobel, M.; McAdory, L. E.; Harris, R. B. *Arch. Biochem. Biophys.* **1996**, *334*, 206−213.
(22) Forgacs, G.; Newman, S. A.; Hinner, B.; Maier, C. W.; Sackmann, E. *Biophys. J.* **2003**, *84*, 1272−1280.
(23) Sanborn, T. J.; Messersmith, P. B.; Barron, A. E. *Biomaterials* **2002**, *23*, 2703−2710.
(24) Elbert, D. L.; Pratt, A. B.; Lutolf, M. P.; Halstenberg, S.; Hubbell, J. A. *J. Controlled Release* **2001**, *76*, 11−25.

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