Rheological Characterization of in Situ Cross-Linkable Hyaluronan Hydrogels

Kaustabh Ghosh,[†] Xiao Zheng Shu,[‡] Robert Mou,[§] Jack Lombardi,[§] Glenn D. Prestwich,[‡] Miriam H. Rafailovich,[||] and Richard A. F. Clark*[,†,⊥]

Departments of Biomedical Engineering, Material Science and Engineering, Dermatology and Medicine, State University of New York at Stony Brook, Stony Brook, New York 11794-8165, Department of Medicinal Chemistry, The University of Utah, 419 Wakara Way, Suite 205, Salt Lake City, Utah 84108-1257, and Physical Chemistry Laboratory, Estee Lauder Research, Melville, New York 11747

Received May 26, 2005; Revised Manuscript Received June 10, 2005

This report investigates the rheological properties of cross-linked, thiol-functionalized HA (HA−DTPH) hydrogels prepared by varying the concentration and molecular weight (MW) of the cross-linker, poly(ethylene glycol) diacrylate (PEGDA). Hydrogels were subsequently cured for either short-term (hours) or long-term (days) and subjected to oscillatory shear rheometry (OSR). OSR allows the evaluation and comparison of the shear storage moduli (G'), an index of the total number of effective cross-links formed in the hydrogels. While the oscillatory time sweep monitored the evolution of G' during in situ gelation, the stress and frequency sweeps measured the G' of preformed and subsequently cured hydrogels. From stress sweeps, we found that, for the hydrogels, G' scaled linearly with PEGDA concentration and was independent of its MW. Upon comparison with the classical Flory's theory of elasticity, stress sweep tests on short-term cured hydrogels revealed the simultaneous, but gradual, formation of spontaneous disulfide cross-links in the hydrogels. Results from time and frequency sweeps suggested that the formation of a stable, three-dimensional network depended strictly on PEGDA concentration. Results from the equilibrium swelling of hydrogels concurred with those obtained from oscillatory stress sweeps. Such a detailed rheological characterization of our HA−DTPH−PEGDA hydrogels will aid in the design of biomaterials targeted for biomedical or pharmaceutical purposes, especially in applications involving functional tissue engineering.

Introduction

Hyaluronan (HA) is a linear, nonsulfated glycosaminoglycan found ubiquitously in the extracellular matrix (ECM) of virtually all mammalian connective tissues.[1] This polyanionic biopolymer is composed of repeating disaccharide units of β-1,4-D-glucuronic acid and β-1,3-*N*-acetyl-D-glucosamine.[2] Previously, HA in tissues was believed to act only as an inert lubricating substance; however, important biological functions of HA are now widely reported in the literature.[1,3] HA occurs naturally in a wide range of molecular weights (MWs; 0.1−10 million) and concentrations[4] and is stabilized by association with a number of link proteins. These attributes impart HA with its unique rheological properties that allow it to fulfill diverse physicochemical functions in different locations in the body. This property of native HA has been exploited therapeutically in viscosupplementation and viscosurgery.[5] However, pure native HA has found limited clinical application, largely due to its poor biomechanical properties and rapid degradation in vivo. To address this problem, several chemical modifications have been successfully employed to significantly improve the biomechanical properties of HA and, thereby, its ease of handling and residence time in vivo.[6−9]

One such novel chemical modification involving the synthesis of thiol-functionalized HA (HA−DTPH) has been previously reported[6] (Figure 1A). By virtue of their ability to form spontaneous disulfide bonds upon exposure to air, the free thiols on HA backbone act as latent cross-linking agents (Figure 1B). Since the formation of disulfide bonds is slow, Michael-type addition between free thiols and acrylates of poly(ethylene glycol) (PEG) has been utilized to rapidly cross-link HA−DTPH. This chemistry, first reported by Lutolf et al.[10] and suitably modified for HA−DTPH,[11] uses homobifunctional PEG diacrylate (PEGDA) to produce an in situ cross-linkable hydrogel in approximately 10 min (Figure 1C). Importantly, the cross-linking reaction between free thiols and PEGDA occurs at physiological pH and room temperature, which ensures complete biocompatibility during cell encapsulation or incorporation of biologically active ligands.[11,13] The disulfide and PEGDA cross-linked HA−DTPH biomaterials (denoted hereafter by HA−S−S−HA and HA−PEGDA−HA, respectively) and their functional derivatives have great potential in drug delivery and tissue engineering applications.[6,11−13]

* Corresponding author: phone 631-444-7519; fax 631-444-3844; e-mail rclark@notes.cc.sunysb.edu.
[†] Department of Biomedical Engineering, SUNY at Stony Brook.
[‡] The University of Utah.
[§] Estee Lauder Research.
[||] Department of Material Science and Engineering, SUNY at Stony Brook.
[⊥] Department of Dermatology and Medicine, SUNY at Stony Brook.

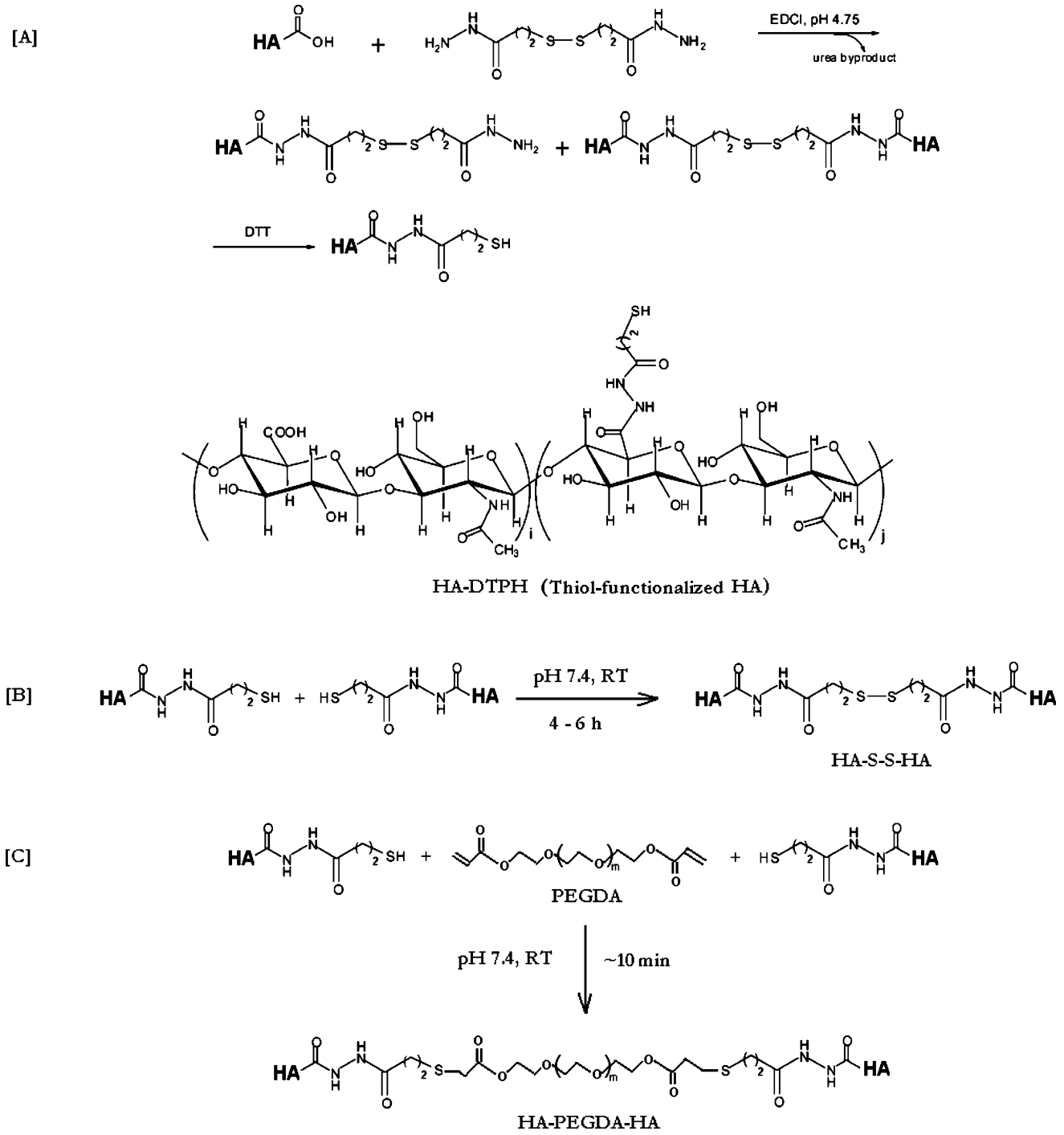

Figure 1. Schematic showing (A) synthesis of thiol-functionalized HA (HA−DTPH),[6] (B) spontaneous cross-linking forming HA−S−S−HA linkages, and (C) exogenous PEGDA-mediated cross-linking forming HA−PEGDA−HA linkages (adapted and modified from Shu et al.[6]).

Previous published data indicate that the rheological properties of most polymeric biomaterials, including HA-based biomaterials, depend not only on the MW and concentration of the macromer but also on the nature and density of effective cross-links.[11,14] Furthermore, the rheological properties of biomaterials can modulate their therapeutic utility. For example, rheological modifications of PLGA film-based implants affect drug release profiles.[15] The cross-linking density influences the stiffness of PEG hydrogels and thereby the synthesis and distribution of extracellular degradation of the resulting hydrogels. The viscoelasticity of cross-linked HA has been shown to affect their therapeutic potential in viscosupplementation,[18] in significant reduction of postsurgical adhesions,[19] and in soft-tissue augmentation.[20] These findings underscore the importance of rheological properties of biomaterials.

This study, therefore, characterizes the rheological properties of the recently reported HA−PEGDA−HA hydrogels.[11] Oscillatory shear rheometry, operated in time, stress, and frequency sweep modes, was used to evaluate the shear

the slow HA−S−S−HA and the rapid HA−PEGDA−HA cross-linking reactions proceed at very different rates,[11] we also monitored G' as a function of short-term (hours) and long-term (days) curing times. Effects of PEGDA concentration and MW on the levels of equilibrium swelling of the various hydrogels were also evaluated.

Materials and Methods

Materials. Fermentation-derived hyaluronan (HA, sodium salt, M_w 1 500 000) was provided by Clear Solutions Biotechnology, Inc. (Stony Brook, NY). 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDCI) and poly(ethylene glycol) diacrylate (PEGDA, MW 700, purity 95%) were purchased from Aldrich Chemical Co. (Milwaukee, WI). PEGDA (MW 3400, purity 98%) was purchased from Nektar Therapeutics (Huntsville, AL). Dulbecco's modified Eagle's medium (DMEM) was obtained from Sigma Chemical Co. (St. Louis, MO). Dithiothreitol (DTT) was purchased from Diagnostic Chemical Limited (Oxford, CT). 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was purchased from Acros (Houston, TX). 3,3′-Dithiobis(propanoic dihydrazide) (DTP) was synthesized as previously described.[6] Distilled phosphate-buffered saline (1× dPBS, pH 7.4) was prepared in the lab according to a standard protocol.

Synthesis of Thiol-Functionalized HA. Thiol-functionalized HA, or 3,3′-dithiobis(propanoic dihydrazide)-modified HA (HA−DTPH), was synthesized according to a previously reported procedure.[6] In principle, the native carboxylic groups on HA disaccharide units were replaced by thiol-containing DTPH groups. The degree of substitution (% SD), defined as the number of DTPH groups per 100 disaccharide units on the HA molecule, was determined by ^{1}H NMR.[6] The free thiol content (percent), defined as the number of free thiols per 100 disaccharide units, was measured in parallel by a modified Ellman method.[6,21,22] Both % SD and the free thiol content were found to be approximately 42%, indicating that the remaining 58% of the HA disaccharide units contained the native carboxylic acid group. The pK_a of thiols in HA−DTPH was 8.87 as determined spectrophotometrically on the basis of UV absorption of thiolates. The MW was determined by calibrated gel-permeation chromatography (GPC) to be M_w 158 000 and M_n 78 000 (polydispersity index 2.03).

Specimen Preparation: Hydrogels. A 1.25% (w/v) HA−DTPH solution was prepared by dissolving HA−DTPH in serum-free DMEM supplemented with 1% (v/v) penicillin, streptomycin, and glutamine (antibiotic mix). The HA−DTPH solution was first pH-adjusted to 7.4 (by addition of 1.0 M NaOH) and then sterilized by filtration through a 0.22 μm filter. A 4.5% (w/v) PEGDA (MW 3400) stock solution was prepared by dissolving PEGDA powder in 1× dPBS. Four volumes of 1.25% HA−DTPH solution were then mixed with one volume of PEGDA solution of varying concentrations (4.5%, 3.0%, 2.25%, 1.5%, and 0.75%) to obtain HA−PEGDA−HA hydrogels of different cross-linking densities (defined in this report as the molar ratio of thiols on HA−DTPH:acrylate groups on PEGDA) of 2:1, ties, HA−PEGDA−HA hydrogels with a cross-linking density of 2:1 were also prepared from PEGDA MW 700. The final concentration of HA−DTPH in the hydrogels was always 1% (w/v). As previously reported,[11] gelation time was found to be inversely proportional to PEGDA concentration. A 1% HA−DTPH solution was also prepared without addition of any PEGDA. All hydrogels were plated in regular 35 mm tissue culture dishes. Serum-free DMEM was added to the surface of all hydrogels to allow for equilibrium swelling prior to oscillatory shear rheometry.

Regardless of the cross-linking densities used in this study, all HA−PEGDA−HA hydrogels ideally contain excess free thiols. We, therefore, investigated the extent of net effective cross-linking in these hydrogels arising from both HA−S−S−HA and HA−PEGDA−HA cross-links as a function of curing time. For this study, all HA−PEGDA−HA hydrogels were plated simultaneously (at $t = 0$); at the end of each experimental time point, an 11 mM iodoacetamide solution in 1× dPBS was added to the hydrogel surface to block residual free thiols and thereby prevent any additional HA−S−S−HA or HA−PEGDA−HA cross-links.

Rheological Characterization: Oscillatory Shear Rheometry of Hydrogels. An AR2000 rheometer (TA Instruments Inc.) with a standard steel parallel-plate geometry of 20 mm diameter was used for the rheological characterization of all hydrogel samples. The test methods employed were oscillatory time sweep, stress sweep, and frequency sweep. The time sweep was performed to monitor, within a given time frame, the in situ gelation of the 2:1, 6:1, and 12:1 HA−PEGDA−HA hydrogel solutions. The strain was maintained at 5% during time sweeps by adjusting the stress amplitude. The test, which was operated at 1 Hz and terminated after 30 min, recorded the temporal evolution of G' and the shear loss modulus, G''.

The stress sweep was performed on hydrogels to determine and compare their G' under the same physical condition. The stress sweep was set up by holding the temperature (25 °C) and frequency (1 Hz) constant while increasing the stress level from 50 to 70 Pa. The applied range of 50−70 Pa was found to be safe-for-use from a prior experiment where we determined the linear viscoelastic region (LVR) profiles of the 2:1, 6:1, and 12:1 hydrogels by shearing them until structure breakdown. In the stress sweep (or "controlled stress") tests, the stress was locally controlled in every cycle and the strain (and the corresponding G') was measured, while globally speaking, the hydrogels were subjected to a steady stress ramp. A constant normal compressional force of $\sim 4g$ was applied to all samples throughout the stress sweep regime. Both the time and stress sweeps provide G' and G'' information on the structural integrity of the cross-linked network, but at two different physical settings.

We also subjected the 2:1, 6:1, and 12:1 hydrogels to a frequency sweep at 50% of their respective ultimate stress levels (corresponding to the point of dip on the LVR profile). At this fixed shear stress and temperature (25 °C), the oscillatory frequency was increased from 0.1 to 100 Hz and the G' was recorded. To avoid dislocation during each test

plots of G' versus shear stress, reaction time, or frequency from the three sweep tests were obtained directly from the software controlling the rheometer. All samples were done in triplicate.

FTIR Analysis. A Nicolet Magna-IR 760 optical bench spectrometer (Thermo Electron Corp.) was used to obtain Fourier transform infrared (FTIR) spectra of pure HA−DTPH, pure PEGDA, and 8-h cured HA−DTPH−PEGDA hydrogel on calcium fluoride disks. A normalized spectrum was obtained by subtracting the HA−DTPH spectrum from the hydrogel spectrum, which was then compared with that of pure PEGDA. The peaks at 1634 and 1410 cm^{-1} (corresponding to −C═C− bond stretching and scissoring, respectively) were used to follow the consumption of PEGDA in the HA−PEGDA−HA hydrogel.

Equilibrium Swelling of Hydrogels. Identical volumes of hydrogel samples were plated in wells of a 24-well plate. All hydrogel samples were allowed to sit at room temperature for about 2 h before weighing them; this allowed the weakly cross-linked 12:1 hydrogels to set well before the start of the experiment. After the initial weights (W_i) were recorded, all hydrogels were gently transferred to weigh boats filled with distilled, deionized water. To obtain equilibrium swelling, all samples were allowed to swell at room temperature for 48 h. The equilibrium swollen mass (W_s) was then recorded by gently blotting excess water from each sample. The hydrogel samples were subsequently dried for 48 h in a desiccator at room temperature and their dry weights (W_d) were measured. The equilibrium swelling ratio (Q) was defined as the ratio of W_s to W_d.

Results and Discussion

Thiol-functionalized HA, or HA−DTPH, is synthesized by substituting the native carboxylic group on HA molecule with free and active thiol groups. Upon exposure to air, the thiols on HA−DTPH are oxidized to form a spontaneous, albeit slow (within 4−6 h), HA−S−S−HA cross-linked network. These HA−S−S−HA cross-links are reversible in nature since addition of DTT (a reducing agent) results in the dissolution of the networked structure.[6] However, to enhance the rate of cross-linking, Michael-type addition reaction is employed where, by use of homobifunctional PEGDA, a HA−DTPH solution is cross-linked to form a stable hydrogel within approximately 10 min.[11] This rapid gelation, also occurring at physiological pH and room temperature, advocates its proposed injectable in vivo use.

In the first published report describing the formation of HA−PEGDA−HA hydrogels,[11] Shu et al. showed an increase in both cross-linking efficiency of PEGDA (i.e., double-end anchorage, from 76.2% to 100%) and the observed gelation time (from 5 to 19 min) with decreasing PEGDA concentration [from 9% to 3% (w/v)]. On the basis of these data, PEGDA concentration of 4.5% (corresponding to a cross-linking density of 2:1) was found to be optimum for use both in vitro and in vivo.[11,13] Importantly, after these optimally cross-linked hydrogels have been formed, about HA cross-links. Both the extent and rate of formation of these disulfide links are likely to alter the microlevel network structure and thereby the rheological and physicochemical properties of these HA−PEGDA−HA hydrogels. Altering the molar concentration and MW of PEGDA can also produce such effects. A similar trend was observed in HA−ADH−PEG dialdehyde hydrogels where changes in PEG dialdehyde (cross-linker) concentration altered the network structure and the inherent physicochemical properties.[23] These issues have, however, never been addressed before for the HA−PEGDA−HA hydrogels. Therefore, in this study we aimed to determine the effect of the above-mentioned parameters on the rheological behavior and levels of equilibrium swelling of the resulting HA−PEGDA−HA hydrogels. On the basis of these data, we propose plausible schemes of cross-linking occurring in these hydrogels.

Rheological Characterization of HA−DTPH Hydrogels. (A) Oscillatory Time Sweep. Oscillatory time sweeps were performed to monitor the in situ gelation of HA−PEGDA−HA solutions prepared from PEGDA MW 3400, the MW shown to be optimal for use both in vitro and in vivo.[11] Figure 2 shows the time sweep profiles of G' and G'' for the 2:1, 6:1, and 12:1 HA−PEGDA−HA hydrogel networks (panels A, B, and C, respectively). Initially, G'' is larger than G', which is expected since the samples are still in liquid state where viscous properties dominate, and therefore most (if not all) of the energy is lost as viscous heat. As the solutions begin to gel and a cross-linked network is formed, both G' and G'' begin to increase; however, the rate of increase of G' is much higher than that of G'' since now the elastic properties of the gelling hydrogel begin to dominate. Consequently, there is a crossover point where G' becomes larger than G''. The time required for this crossover to occur is sometimes referred to as the gelation time for the solution.[24] Although the apparent gelation times observed by the "test tube inverting" method were greater than those observed in these profiles, they were proportionate for all three cross-linking densities. Furthermore, from Figure 2 we see that with increased PEGDA concentration the crossover point appears sooner, indicating that PEGDA cross-linking of HA−DTPH is the rate-limiting reaction during this early phase of gelation. Although the plot of G'' plateaus with time, it never decreases to 0, suggesting the viscoelastic nature of these hydrogels under the applied physical conditions. The slightly erratic nature of G'' observed during the time sweep tests is attributed to grip-slip caused by the release of water from the hydrogels as they undergo shear stress.

(B) Oscillatory Stress Sweep. Oscillatory stress sweep allows determination of G' of the hydrogels as a function of PEGDA concentration and MW. The effect of curing time on G' can also be similarly evaluated. The data obtained can be further used to predict and compare the rate and extent of formation of effective cross-links in various hydrogels. In compliance with the principle of small deformation rheology,[24] the hydrogels must be tested within their respective linear viscoelastic ranges, the length of which determines the structural stability. We, therefore, first determined the

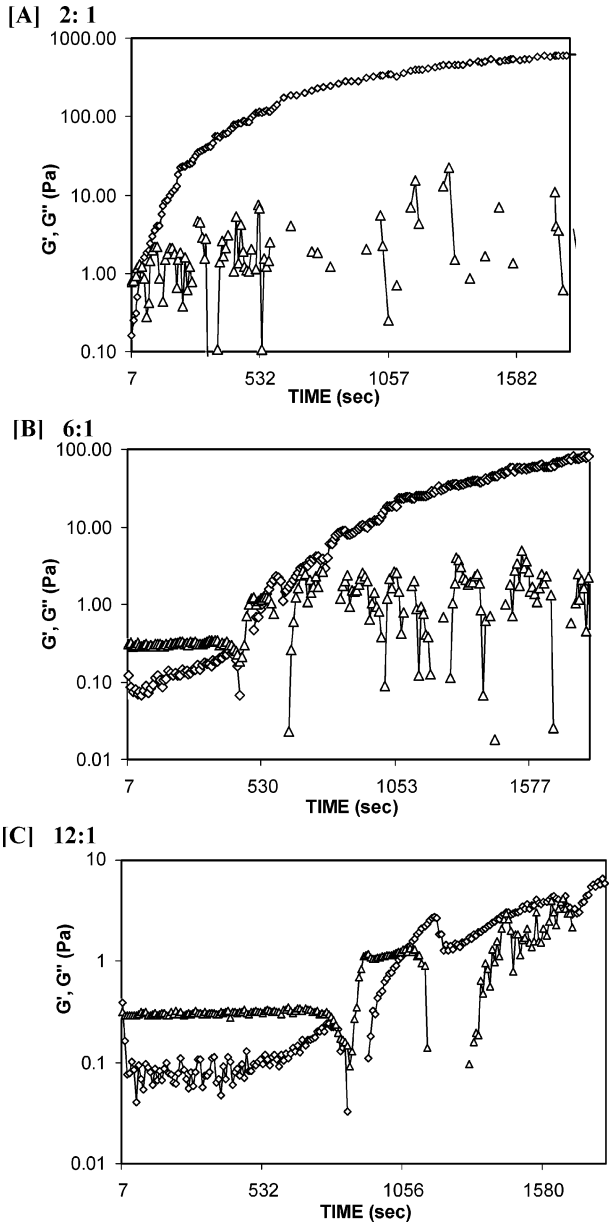

Figure 2. Evolution of shear storage moduli, G' (◇), and shear loss moduli, G'' (△), as a function of time during the pregelation and early gelation phases of (A) 2:1, (B) 6:1, and (C) 12:1 HA−PEGDA−HA hydrogels (PEGDA MW 3400).

breakdown. Since the HA−PEGDA−HA and HA−S−S−HA cross-links, and the corresponding rheological properties, are expected to develop over varying time scales (minutes to hours to days),[11] it becomes impractical to perform rheological tests at all time points. Therefore, rheological properties such as the LVR profile and frequency response of G' (in the following section) were determined at a fixed curing time of 8 h. The choice of this curing time point was justified from previous studies on a similar system (HA−PEG monoacrylate conjugation)[11] that intuitively suggest that HA−PEGDA−HA cross-links might more or less reach completion within this period. Figure 3 represents the LVR profile of the 8-h cured HA−PEGDA−HA hydrogels, showing clearly that, with increasing cross-linking density, the structure breakdown occurred at higher shear stress levels.

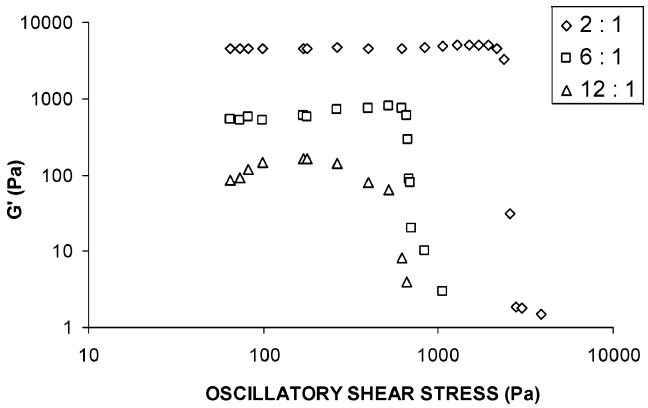

Figure 3. Determination of the linear viscoelastic region (LVR) of the 8-h cured 12:1, 6:1, and 2:1 cross-linked HA−PEGDA−HA hydrogels (PEGDA MW 3400). Frequency of the applied oscillatory shear stress was 1 Hz.

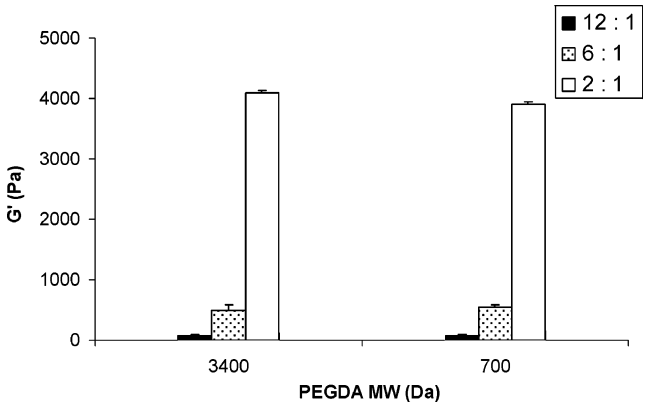

Figure 4. Comparison of G' of HA−PEGDA−HA hydrogels prepared from PEGDA of MW 3400 and 700. Oscillatory shear stress was performed at 1 Hz.

verified to be lying in the LVR of even the least (2 h) cured hydrogels (data not shown).

We next looked at the effect of PEGDA MW on the storage moduli of the hydrogels. PEGDA MW 700 and 3400 were used to prepare 2:1, 6:1, and 12:1 HA−PEGDA−HA hydrogels. These hydrogels were subsequently cured for 8 h before being subjected to stress sweep. Figure 4 illustrates that G' of HA−PEGDA−HA hydrogels was almost entirely independent of PEGDA MW; G' was, however, strongly dependent on the number of cross-links formed, which was controlled by PEGDA concentration. We inferred that, in a hydrated state, water molecules act as the most flexible component and therefore the hydrogel stiffness becomes insensitive to the flexibility imparted by the length (or MW) of the PEGDA molecule. As a result, all subsequent oscillatory stress sweeps were performed on hydrogels prepared from PEGDA MW 3400 only.

Next, hydrogels of varying cross-linking densities were formed and allowed to cure for 2, 4, 8, 10, and 24 h. Existing theories and numerous published reports have earlier suggested a strong correlation between the measured G' and the number of effective intermolecular cross-links formed in a hydrogel network.[14,15,17,25,26] We, therefore, monitored and compared the evolution of G' as a function of cross-linking density and curing time to assess the extent of effective

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.