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The role of heparin self-association in the gelation of heparin-functionalized polymers

Frances J. Spinelli^a, Kristi L. Kiick^{b,c}, and Eric M. Furst^{a,*}

^aDepartment of Chemical Engineering, University of Delaware, 150 Academy Street, Colburn Laboratory, Newark, DE 19716, USA

^bDepartment of Materials Science and Engineering, University of Delaware, 201 Dupont Hall, Newark, DE 19716, USA

^cDelaware Biotechnology Institute, University of Delaware, 15 Innovation Way, Newark, DE 19711, USA

Abstract

The extensive use of the glycosaminoglycan (GAG) heparin in the design of emerging biomaterials has made the physical characterization of this heterogeneous biomacromolecule increasingly important. In this work, heparin solutions are characterized via dynamic light scattering to investigate heparin's self-association, since this behavior was recently hypothesized to play a role in the gelation of heparin-functionalized polymer hydrogels. Samples of either low molecular weight heparin or high molecular weight heparin were filtered using membranes with 100, 220, or 450 nm average pore sizes. The 100 and 220 nm filters produce a single population of monomers with a diameter range of 3–10 nm in the intensity-weighted size distribution. However, the 450 nm filters reveal a second population of associated heparin. Increasing the solution concentration of high molecular weight heparin (HMWH) from 2.5 to 10 wt% causes the magnitude of the smaller population to decrease, while the diameter of the larger associated species approximately doubles. HMWH from different manufacturers displays varying degrees of association. Therefore, weaker associating HMWH can potentially be identified to control heparin self-interactions. Finally, fractionated, *N*-deacetylated low molecular weight heparin (LMWH) is compared to unmodified LMWH. The chemically modified heparin exhibits a heightened degree of association, suggesting an enhanced self-interaction. The increased negative charge of LMWH in the fractionated sample likely enhances polyelectrolyte interactions proposed to drive the association of these similarly charged polysaccharides. A more detailed understanding of heparin–heparin interactions will assist in the design of new scaffold materials with controlled release profiles, in the clinical use of heparin as an anticoagulant, and in investigations of interactions of other like-charged biomacromolecules.

Keywords

Hydrogel; Heparin; Mechanical properties; Rheology; Cross-linking

1. Introduction

Heparin is a biologically relevant, highly anionic polysaccharide. The biological roles of this glycosaminoglycan (GAG) have been widely studied due to its importance in medicine and biomedical applications, including its common clinical use as an anticoagulant. Heparin is known to reversibly bind to many biofunctional proteins, such as antithrombin III (ATIII),

*Corresponding author. Tel.: +1 302 831 0102; fax: +1 302 831 1048. E-mail address: furst@udel.edu (E.M. Furst).

heparin interacting protein (HIP), and platelet factor 4 (PF4) [1]. In addition, heparin contains distinct recognition sites for growth factors, such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) [1].

Various heparins are distinguished by their average number of disaccharide motifs, although all carry many sulfate and carboxyl groups. Heparin behaves as a linear polyelectrolyte with one of the largest negative charge densities of any biomacromolecule [1]. High molecular weight heparin (HMWH) demonstrates polydispersity in both composition and length, and typically consists of 25 disaccharide residues, with a molecular weight range of 5–40 kDa and an average charge of -75 [1,2]. Similarly, low molecular weight hepar (LMWH) also displays heterogeneity, with a molecular weight range of 1–10 kDa, corresponding to on average approximately 5 disaccharide residues.

Heparin's ability to associate with a wide variety of proteins makes it an ideal candidate as a noncovalent cross-linker and drug delivery agent in hydrogels with controlled release profiles [3–6]. Accordingly, it has been incorporated into noncovalently assembled, polymeric hydrogel networks based on its interactions with known heparin-interacting peptides and proteins [3–12]. Owing to the use of heparin, these polymer matrices also have the ability to deliver growth factors, such as bFGF and VEGF, to cells in a controlled fashion, to encourage cell migration into and through the artificial scaffold. Poly(ethylene glycol) (PEG) has been used as the polymeric backbone in these noncovalently assembled networks due to its known biocompatibility, hydrophilicity, and lack of protein fouling [13,14].

For example, Seal and Panitch described formation of viscoelastic networks comprising a 4-arm star PEG chemically attached to heparin-binding peptides (HBP), such as sequences from ATIII and TAT, that are cross-linked with HMWH in solution [7]. These networks also have the ability to bind and release other heparin-binding molecules. A second hydrogel network incorporates cross-linker peptides that chemically connect 8-arm star PEG, in addition to the physical junctions provided by multifunctional HMWH [8,9]. Furthermore, Benoit and coworkers have copolymerized methacrylated HMWH and dimethacrylated PEG to form gels that sequester bFGF and promote attachment and differentiation of human mesenchymal stem cells (hMSCs) [10,11]. In other studies, LMWH was employed by Yamaguchi et al. to minimize the chemical cross-linking of multiple 4-arm star PEG termini with a single heparin molecule, yielding soluble, 4-arm star PEG-LMWH conjugates [3,4]. Physical networks were formed by mixing this PEG-LMWH with heparin-binding polymers comprising heparin-binding peptides (HBP) conjugated to PEG 4-arm stars [3,4]. In addition, physical networks have also been formed through the direct association of similar PEG-LMWH conjugates with dimeric, heparin-binding growth factors (specifically VEGF) [5]. This hydrogel degrades through receptor-mediated erosion as VEGF is delivered to cells.

Unexpectedly, Yamaguchi et al. found that certain PEG-LMWH solutions could form hydrogels in the absence of the PEG-HBP conjugate [3]. The gelation of PEG-LMWH was attributed to the self-association of heparin [15–17]. There have been previous, albeit limited, reports of such self-associative behavior. For instance, dynamic light scattering (DLS) was employed by Gaigalas et al. to characterize the size and charge heterogeneity of commercial preparations of heparin. Populations of slow and fast diffusing species were reported. The larger aggregates could be removed through filtration (0.1 μm pore size), and were stable up to a temperature of 75 $^{\circ}\text{C}$, suggesting that they are composed of tightly bound heparin molecules. The cause of this apparent aggregation was not discussed in detail, nor studied further. The reproducibility of pharmacokinetic activity in clinical heparin treatments and of physical properties of heparin-based biomaterials may be considerably influenced by the existence of such aggregated species [15].

In this work, we present characterizations of heparin self-interactions using dynamic light scattering. Several forms of heparin relevant to their application in biofunctional hydrogels are investigated, including both high and low molecular weight heparin as well as fractionated, *N*-deacetylated LMWH, which is most chemically similar to the heparin employed previously in PEG–LMWH hydrogels [3,4]. In addition, two heparin sources are also compared. Our results support the concept of heparin self-association previously reported by Gaigalas and coworkers [15], as well as the heparin interactions observed by Yamaguchi and coworkers in the formation of PEG–LMWH hydrogel networks [3]. We propose that the heparin association is caused by a polyelectrolyte interaction, similar to other highly charged biomacromolecules including DNA, xanthan, and tobacco mosaic virus (TMV) [18–22].

2. Methods

2.1. Materials and sample preparation

High molecular weight heparin sodium salt (HMWH, porcine intestinal mucosa, $M_r = 15\,000$) and low molecular weight heparin sodium salt (LMWH, porcine intestinal mucosa, $M_r = 3000$) were obtained from Sigma–Aldrich (St. Louis, MO) or Acros Organics (Geel, Belgium). Heparin samples were dissolved in Dulbecco's phosphate buffered saline (PBS 1X, Invitrogen Corp., Carlsbad, CA) and vortexed for 30 s. Several types of heparin were studied, including HMWH (Sigma and Acros), unmodified LMWH (Sigma), and chemically modified LMWH that was fractionated and *N*-deacetylated [4]. Several parameters were tested, including the effects of filtration, concentration, and time. Samples were filtered directly into the scattering cell (ampule, Wheaton, Millville, NJ) via use of 100, 220, or 450 nm pore size syringe driven filter units from Millipore (Low Protein Binding Durapore, Carrigtwohill, Co. Cork, Ireland). The heparin concentration was varied from 2.5 to 10 wt% in studies of concentration-dependent aggregation and was held constant at 3.3 wt% to compare chemical species and manufacturers. Final heparin concentrations after filtration were verified using toluidine blue assays, as previously reported [4,23].

2.2. Synthesis of *N*-deacetylated, fractionated LMWH

The synthesis of *N*-deacetylated, fractionated LMWH was performed as previously described [4]. Briefly, low molecular weight heparin (LMWH) was fractionated on an anionic exchange chromatography DEAE resin into low- and high-affinity fractions. The high-affinity fraction was then *N*-deacetylated by treatment with hydrazine [24]. The final product was dialyzed against 1 M NaCl followed by ultrapure water (18 MΩ cm, four changes each) and lyophilized before reconstituting for use in the experiments.

2.3. Dynamic light scattering (DLS)

Heparin dissolved in PBS was characterized via DLS using a Brookhaven correlator and goniometer (BI-9000). All measurements were performed for 10 min at a scattering angle of 90° at 25 °C, and were obtained immediately after solution preparation. Analysis of the intensity autocorrelation function was conducted via the CONTIN method, generating intensity-weighted size distributions [25,26]. Additional measurements were made several hours and days after sample preparation in an attempt to determine the kinetics of association of heparin monomers into aggregates. During the incubation time, samples were stored at 4 °C and were warmed to room temperature under ambient conditions prior to performing light scattering. To confirm the consistency of the results, light scattering experiments were conducted at several scattering angles, including 60°, 90°, and 120°. Each sample was analyzed at least twice, and every experimental condition was duplicated to ensure reproducibility.

2.4. Toluidine blue assay

Toluidine blue assays were employed as previously reported to identify the final concentration of heparin in solution after filtration [23]. A calibration curve was constructed from absorbance measurements at 631 nm of dilute, aqueous heparin solutions in 0.005% toluidine blue (Aldrich, Milwaukee, WI) and 0.2% NaCl, after organic extraction of the heparin–toluidine blue complex. Heparin solutions were vortexed and filtered in the same manner and at the same concentration as light scattering samples were prepared. The samples were diluted by a factor of 1000 after filtration to yield concentrations in the range of the standard calibration curve. To determine the amount of heparin in solution after filtration, the diluted heparin solutions (0.1 mL), 0.2% NaCl (2.4 mL), and 0.005% toluidine blue (1 mL) were vortexed for 30 s prior to and after the addition of hexane (3 mL). Organic extraction of the heparin–toluidine blue complex was allowed for 12 h. The amounts of heparin in solution before and after filtration were calculated from the absorbance at 631 nm of the aqueous phase of samples of unknown heparin concentration as compared to the standard calibration curve.

3. Results

3.1. Filtration and concentration effects on HMWH interactions

Fig. 1 shows DLS results for 2.5, 5, and 10 wt% HMWH solutions eight days after filtering with 100 or 450 nm average pore size filters. All heparin concentrations, after filtration through a 100 nm pore size filter, exhibit intensity autocorrelation functions with only a single exponential decay. This indicates that only the fast-diffusing monomer species is present, with a diameter between 3 and 10 nm, in agreement with previously reported radius of gyration values of $3.2\text{--}5.5 \pm 0.2$ nm (Fig. 1A, C) [2]. In contrast, upon filtration with a larger pore size filter (450 nm), two components are present, the fast-diffusing monomers and a slow-diffusing species, as evidenced by the multiple exponential decays of the correlation function (Fig. 1B, D). The first exponential decay is again consistent with the monomer species, while the second corresponds to larger diffusive species with diameters between 40 and 1000 nm. The concentration dependence of the size distributions is strongly governed by the filter size. For the 100 and 220 nm filtrations, no change in the light scattering is observed when the concentration is varied between 2.5 and 10 wt%. In contrast, for samples filtered with 450 nm pore size membranes, an increase in HMWH concentration from 2.5 to 10 wt% results in a decrease in the magnitude of the monomer peak, while the aggregate population size increases in diameter (Fig. 1). After eight days, the larger population exhibits diameters in the range of 40–300, 100–300, and 300–1000 nm for 2.5, 5, and 10 wt% HMWH, respectively.

3.2. Chemical variation of heparin

The effects on heparin association of chemical composition and manufacturer were compared at a constant heparin concentration of 3.3 wt% (Fig. 2 and Fig. 3). All samples filtered with 100 and 220 nm average pore size showed a single monomer species with a diameter of 3–10 nm; the size of this species was stable for over six weeks. After 450 nm filtration and an eight-day incubation time, the fractionated, *N*-deacetylated LMWH species displayed three distinct populations: the monomer (3–10 nm), as well as two larger species, with sizes ranging between 100 and 1000 nm and in excess of 2000 nm (Fig. 2A, C). Similarly, upon filtration with a 450 nm pore size membrane, unmodified LMWH solutions initially had two populations (Fig. 2B, D Day 1), which over nine days grew to three populations (Fig. 2B, D Day 9) of similar size to those observed for the modified LMWH (Fig. 2A, C), but significantly lower in magnitude. This indicates that unmodified LMWH exhibits a significantly lower degree of association. High molecular weight heparin from Sigma exhibited a stronger contribution from aggregates to the light scattering than heparin obtained from Acros (Fig. 3). The monomer population in the Sigma HMWH sample has a lower magnitude than the Acros HMWH solution, and its

aggregated species (with diameters in the 50–1000 nm range) has a larger magnitude of scattering than the Acros HMWH solution.

3.3. Heparin association over time

Heparin samples, which initially contain purely monomers after treatment with 100 nm average pore size filters, did not show any sign of aggregation over six weeks (Fig. 4). In contrast, samples filtered with the 450 nm average pore size show evidence of aggregate growth over an eight-day period (Fig. 1B and Fig. 2). To verify the reliability of these results, light scattering experiments were performed at multiple scattering angles including 60°, 90°, and 120° (Fig. 5). All measurements indicate similar distributions of sizes, confirming the presence of the larger associated populations.

3.4. Heparin concentrations after filtration

Toluidine blue assays of heparin concentration before and after filtration confirmed that the heparin concentration did not change significantly upon filtration at the weight percents studied here. Fig. 6 displays the assay results for 3.3 wt% HMWH (Sigma) diluted to 33 µg/mL after filtration. Heparin solutions filtered with 100, 220, and 450 nm average pore size filters were within the experimental variation of the unfiltered sample.

4. Discussion

From dynamic light scattering measurements, it has been shown that heparin interacts to form aggregated species. This self-association is also apparent in the formation of PEG–LMWH hydrogel networks where LMWH has been modified and attached to star PEG, and itself acts as a noncovalent cross-linker [3,5]. Dynamic light scattering experiments confirm our hypothesis, indicating the existence of several distinct species of various sizes in solutions of heparin, in both monomer and aggregate forms (Fig. 1B, Fig. 2 and Fig. 3). The monomer species shows diameters in the range of 3–10 nm, in agreement with small angle X-ray scattering (SAXS) measurements performed by Pavlov and coworkers that indicated values of radius of gyration between 3.2 and 5.5 ± 0.2 nm [2]. The range of aggregate diameters is of the same magnitude as values previously reported by Gaigalas et al. between 10^2 and 10^3 nm [15]. Our results suggest that the associating fraction of heparin is already present as aggregates in the sample solution as prepared, and these aggregates are removed through filtration with both 100 and 220 nm filters. This suggests that (1) the heparin interactions are substantially strong and (2) the aggregates are larger than 220 nm, which is confirmed by the size distributions that show aggregate diameters from 10^2 to 10^3 nm (Fig. 2, Fig. 3 and Fig. 5). In addition, the presence of multiple heparin populations agrees with work published by Gaigalas et al., which also showed stable, filterable heparin aggregates [15].

After using filtration to isolate monomer species, heparin molecules remained as suspended monomers over eight days. In solutions filtered with 450 nm pore size membranes, aggregates already present in solution grew in size over the same period, suggesting that the aggregate species may facilitate a cooperative association (Fig. 1 and Fig. 2). This aggregation appears to consume monomer, as indicated by the reduction in the magnitude of scattering from monomers in solution over an eight-day incubation period, in the solutions containing aggregates (450 nm filter). The further association of heparin only in solutions with aggregates already present suggests that the heparin monomers may have slower kinetics or weaker forces of association, possibly due to salt present in the buffer that screens electrostatic and polyelectrolyte interactions [18–22]. Alternatively, the monomer concentration may not be sufficiently high to facilitate aggregation. An initial concern was that filtration with the small pore size membrane may have drastically reduced the heparin concentration in solution, diluting the concentration below a threshold necessary to form aggregates. Toluidine blue

assays of the heparin concentration after filtration were therefore performed, and showed that the heparin concentration does not change significantly upon filtration at the weight percents studied (Fig. 6) [23]. At both 2.5 and 3.3 wt% HMWH (from Sigma and Acros) after 450 nm pore size filtration, the aggregate species are stable over time, with a diameter range of 40–300 nm, but at 5 and 10 wt% HMWH (Sigma) the aggregates grow to 100–300 and 300–1000 nm, respectively, suggesting a potential threshold concentration for aggregate association between 3.3 and 5 wt% HMWH, or that the fraction capable of aggregating has been depleted in the solutions of lower concentration to levels that do not promote additional aggregation (Fig. 1B and Fig. 3).

Fractionated, *N*-deacetylated low molecular weight heparin is the most chemically similar to the material used previously in PEG–LMWH hydrogel networks [3,4]. This chemical modification of LMWH increased the weighting of CONTIN intensity of the larger species, suggesting that the heparin interactions were altered (Fig. 2). It is likely that this enhanced self-interaction is caused by the separation of LMWH into a high-affinity fraction with higher net negative charge. In hydrogel formation, such fractionation was desirable for enhancing the interactions with positively charged peptides designed as noncovalent cross-linkers [4]. In these studies, the increased charge of modified LMWH enhances heparin–heparin interactions and supports the hypothesis that the primary mechanism of association is caused by a polyelectrolyte interaction [18–22]. This polyelectrolyte interaction has been examined in the presence of high salt concentration and at elevated temperatures. Previous work has shown that the addition of 2 M NaCl and 0.03 M EDTA to heparin solutions has no effect on heparin association, as the aggregates were still present at high salt concentrations [15]. Furthermore, the associated species displayed high thermal stability up to 75 °C.

The impact of the commercial source of heparin on the aggregation of heparin was also investigated by sampling HMWH from Sigma–Aldrich and Acros Organics. Heparin from both sources, at 3.3 wt% HMWH filtered with a 450 nm pore size filter, exhibited stable species with populations at both 3–10 nm and 100–600 nm; HMWH from Sigma, however, showed a higher fraction of aggregates and a smaller population of monomers compared to HMWH from Acros (Fig. 3). Therefore, this information can be implemented to more rationally engineer hydrogel properties, and the use of Acros heparin in heparin-based gels would reduce heparin–heparin physical cross-links. HMWH solutions were characterized on DEAE columns in an attempt to quantify the amount of aggregates in solution, but the fraction of aggregated heparin proved to be too low in concentration to quantify via UV-VIS detection, likely due to its small concentration, and in agreement with toluidine blue assay estimates of interactions from rheological measurements of PEG–heparin hydrogels (data not shown, Fig. 6).

The cross-link density in PEG–LMWH gel networks, N , was estimated from the storage modulus obtained from rheological measurements previously performed by Yamaguchi and coworkers [3,5], by employing the relation $G'_{\omega=0} = Nk_bT$. This experimentally calculated cross-link density was then compared to the theoretical maximum density estimated from the total number of available heparin sites in the networks. The specific PEG–LMWH containing networks studied here were made from *N*-deacetylated LMWH that was either fractionated on an anionic exchange column (collecting the most negative fraction) or was unfractionated. Table 1 summarizes the calculated cross-link densities for 2.5 and 7.1 wt% PEG–fractionated LMWH, as well as 8 wt% PEG–unfractionated LMWH. In all cases, the theoretical maximum density exceeds the experimental density by several orders of magnitude. The PEG–fractionated LMWH gels exhibit 0.35 and 0.47% cross-linking for 2.5 and 7.1 wt% gels with moduli of 16.1 and 61.4 Pa, respectively, while the PEG–unfractionated LMWH gels have a lower modulus of 0.7 Pa, demonstrating decreased cross-linking of 0.0048% for a 8.0 wt% gel. These results are consistent with the increased number of aggregates and increased aggregate

size observed in the fractionated LMWH relative to the unfractionated LMWH, and indicate the potential impact of the aggregation on the physical properties of heparinized materials.

In conclusion, the formation of these heparin aggregates may be caused by strong polyelectrolyte interactions between like-charged macromolecules [18–22]. Similar to reports of DNA, tobacco mosaic virus (TMV), and xanthan self-interactions, heparin is another example of a highly negative, linear biological macromolecule that displays strong self-interactions [19,20]. Interestingly, we have found that only a small fraction of heparin actively interacts as estimated from previous rheological measurements [3,5]. The cross-linking density calculated from rheological data is 0.0048–0.47% of the maximum density estimated from the available heparin in the networks (Table 1). This suggests that a limited amount of heparin is interacting to form PEG–LMWH hydrogel networks, as seen from the low storage moduli between 0.7 and 61.4 Pa. Furthermore, conjugates made with fractionated LMWH have an increased storage modulus as compared to unmodified heparin, with $G'_{\omega=0}$ at 61.4 and 0.7 Pa for 7.1 and 8.0 wt% fractionated and unfractionated LMWH, respectively. The fractionated heparin associates two orders of magnitude more than the unfractionated heparin, as indicated by the comparison of the estimated 0.48 to 0.0047% of heparin that actively cross-links, respectively. This agrees with our DLS measurements that show that fractionated heparin has a greater proportion of associating species as compared to unfractionated heparin (Fig. 2). The fraction of aggregated species in the light scattering measurements may appear to be larger than the percentages estimated here from the rheology but we must remember that the light scattering distributions are composed of weighted populations where the larger particles display a increased intensity. In addition, the removal of aggregated heparin after filtration is not measurable through toluidine blue assays (Fig. 6). This is consistent with the very small fraction of associating heparin indicated by rheology; removal of this small fraction is outside the accuracy of the toluidine blue experiments. Fast protein liquid chromatography (FPLC) experiments were unsuccessful in separating the associating heparin on a DEAE column which also confirmed that the aggregated species represent only a small percentage of the heparin population (data not shown).

5. Conclusions

These studies demonstrate that heparin exhibits a tendency for association that varies based on its chemical composition. Dynamic light scattering experiments show multiple populations of heparin in both monomeric and associated forms at diameters of approximately 3–10 and 10^2 – 10^3 nm, respectively. Filtration with 100 and 220 nm average pore size filters removes the associated species, while 450 nm filtration does not. At concentrations above 3.3 wt%, the associated species increase in size over eight days, whereas samples with only monomer present after filtration do not exhibit aggregate formation over the same time period. The association is manufacturer-dependent, which offers opportunities to modulate heparin association in biomaterials. Chemically modified heparin that has been fractionated and *N*-deacetylated possesses the most similar chemical composition as the heparin used in PEG–LMWH hydrogels [3,4]. This modified LMWH demonstrates an amplified heparin self-interaction, potentially caused by its increased negative charge. This is also confirmed by previously reported rheological measurements. The enhanced self-interaction with an increased negative charge supports our hypothesis that heparin association is mediated by a polyelectrolyte interaction, similar to other polyelectrolyte biomolecules. The ability to characterize and control heparin interactions has importance in the applications of this biomolecule both clinically as an anticoagulant and in the design of new biomaterials.

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Fig. 1. Filtration and concentration effects on the association of HMWH in solution. (A, B) Intensity autocorrelation functions and (C, D) intensity-weighted size distributions for the comparison of 2.5 (circles), 5 (squares), and 10 (triangles) wt% HMWH (Sigma) samples upon filtration with (A, C) 100 nm and (B, D) 450 nm pore size filters after an eight-day incubation time.

Fig. 2. Effect of chemical variation on the association of LMWH in solution. (A, B) Intensity autocorrelation functions and (C, D) intensity-weighted size distributions of 3.3 wt% solutions of (A, C) fractionated, *N*-deacetylated LMWH (Sigma) eight days after filtration with 100 (circles) and 450 (triangles) nm pore size filters; and (B, D) unmodified LMWH (Sigma) one day (squares) and nine days (inverted triangles) after 450 nm filtration.

Fig. 3.

Comparison of the association of Sigma and Acros supplied HMWH in solution. (A) Intensity autocorrelation functions and (B) intensity-weighted size distributions of 3.3 wt% solutions of HMWH from Sigma (circles) and Acros (squares) after filtration with 450 nm pore size filters. The size distributions remained constant after 17 days (data not shown).

Fig. 4.

Association of monomeric heparin in solution over time. Data are shown for fractionated, *N*-deacetylated LMWH solutions several weeks after filtration with 100 nm pore size filters. (A) Intensity autocorrelation functions and (B) intensity-weighted size distributions of 3.3 wt% LMWH at 8 (circles), 36 (squares), and 43 (triangles) days after sample preparation.

Fig. 5. Light scattering measurements at multiple scattering angles. Data are shown for fractionated, *N*-deacetylated LMWH solutions after filtration with 450 nm pore size filters. (A) Intensity autocorrelation functions and (B) intensity-weighted size distributions of 3.3 wt% LMWH at 60 (circles), 90 (squares), and 120 (triangles) degrees at 44, 36, and 44 days after sample preparation, respectively.

Fig. 6. Heparin concentrations after filtration. Toluidine blue assays were used to determine the heparin concentration before and after filtration. Shown here is the analysis of 3.3 wt% HMWH (Sigma) diluted to 33 µg/mL after filtration. Heparin solutions filtered with 100, 220, and 450 nm average pore size filters were within the experimental variation of the unfiltered sample.

Table 1

Cross-link density estimation

wt%	Fractionated	$G_{0=0}$ (Pa)	$N_{\text{exp}}/\text{m}^3$	$N_{\text{max}}/\text{m}^3$	% Cross-linked
2.5	Yes	16.1	3.92×10^{21}	1.11×10^{24}	0.35
7.1	Yes	61.4	1.49×10^{22}	3.16×10^{24}	0.47
8.0	No	0.7	1.70×10^{20}	3.56×10^{24}	0.0048

The number of effective cross-link sites per cubic meter in PEG-LMWH gels was calculated from rheological data and compared to the maximum number of cross-linkable sites [3,5].