

Role of Glycosaminoglycan Sulfation in the Formation of Immunoglobulin Light Chain Amyloid Oligomers and Fibrils*

Received for publication, June 1, 2010, and in revised form, August 31, 2010. Published, JBC Papers in Press, September 24, 2010, DOI 10.1074/jbc.M110.149575

Ruiyi Ren^{‡§}, Zhenning Hong^{‡¶}, Haiyan Gong^{||**}, Kate Laporte[§], Martha Skinner^{§††}, David C. Seldin^{§††}, Catherine E. Costello^{‡§¶}, Lawreen H. Connors^{‡§}, and Vickery Trinkaus-Randall^{‡§***1}

From the Departments of ^{||}Anatomy and Neurobiology, [‡]Biochemistry, ^{††}Medicine, and ^{**}Ophthalmology, [§]the Amyloid Treatment and Research Program, and the [¶]Mass Spectrometry Resource, Boston University School of Medicine, Boston, Massachusetts 02118

Primary amyloidosis (AL) results from overproduction of unstable monoclonal immunoglobulin light chains (LCs) and the deposition of insoluble fibrils in tissues, leading to fatal organ disease. Glycosaminoglycans (GAGs) are associated with AL fibrils and have been successfully targeted in the treatment of other forms of amyloidosis. We investigated the role of GAGs in LC fibrillogenesis. *Ex vivo* tissue amyloid fibrils were extracted and examined for structure and associated GAGs. The GAGs were detected along the length of the fibril strand, and the periodicity of heparan sulfate (HS) along the LC fibrils generated *in vitro* was similar to that of the *ex vivo* fibrils. To examine the role of sulfated GAGs on AL oligomer and fibril formation *in vitro*, a κ 1 LC purified from urine of a patient with AL amyloidosis was incubated in the presence or absence of GAGs. The fibrils generated *in vitro* at physiologic concentration, temperature, and pH shared morphologic characteristics with the *ex vivo* κ 1 amyloid fibrils. The presence of HS and over-*O*-sulfated-heparin enhanced the formation of oligomers and fibrils with HS promoting the most rapid transition. In contrast, GAGs did not enhance fibril formation of a non-amyloidogenic κ 1 LC purified from urine of a patient with multiple myeloma. The data indicate that the characteristics of the full-length κ 1 amyloidogenic LC, containing post-translational modifications, possess key elements that influence interactions of the LC with HS. These findings highlight the importance of the variable and constant LC regions in GAG interaction and suggest potential therapeutic targets for treatment.

Amyloid deposition is hypothesized to play a role in many diseases including rheumatoid arthritis and diabetes, as well as the amyloidoses themselves (1). The amyloidoses are a group of systemic and localized diseases that exhibit deposition of insoluble fibrillar proteins in organs and tissues. Primary amyloidosis (AL)² is the most common systemic form in the United

States. It results from a plasma cell dyscrasia in which clonal B cells produce an excess of amyloidogenic immunoglobulin light chains (LCs), which circulate and deposit as insoluble fibrils throughout the body (2). A structural rearrangement from non-fibril-prone oligomers to more fibril-prone conformers is required for protofilament formation (3). Nascent protofilaments can nucleate and elongate through the addition of partially folded oligomeric intermediates to the ends of the growing protofilaments. Protofilaments can further intertwine to form protofibrils and fibrils (4, 5).

Glycosaminoglycans (GAGs) are one of the major components of the extracellular matrix. They consist of disaccharide repeating units that are unbranched and attached to a serine residue of a core protein through a trisaccharide linker. Each GAG has a unique sequence, and post-assembly modifications, which can be induced by a number of factors, lead to alterations in sulfation, acetylation, or length of chain (6). Recently, Staples *et al.* (7) showed that the function of heparan sulfate (HS) might be regulated by its structure and the sulfation at the non-reducing end.

Evidence for the *in vivo* relationship between GAGs and amyloid deposits has been provided by a number of investigators (8, 9). Cardiac tissue containing amyloid deposits has been reported to have a 5-fold increase in GAGs (10). This initial report was substantiated by the observations of Nelson *et al.* (11), who detected GAGs in purified preparations of both amyloid-associated fibrils and AL fibrils. Kisilevsky and co-workers (12, 13) hypothesized that the deposition of truncated serum amyloid A (SAA) was preceded by the up-regulation of HS and chondroitin sulfate (CS) proteoglycans. Interestingly, other investigators demonstrated that HS promoted the aggregation of SAA (14) and hypothesized that HS was a stabilizer of amyloid (15). The association of GAGs with other fibrillar deposits was demonstrated by Cohlberg *et al.* (16), whose studies showed that heparin increased the rate of fibrillation of α -synuclein and actually became an integral part of the fibril. In addition, we have demonstrated that when cardiac fibroblast cells were exposed to AL LCs, the LCs were internalized and the cells then secreted highly sulfated GAGs (17). Together, these data indicate that GAGs and amyloid fibrils have closely related interactions. Thus, a better understanding of the interaction of

* This work was supported, in whole or in part, by National Institutes of Health Grants P01 HL-068705 (to M. S.), EY06000 (V. T.-R.), and P41 RR10888 (C. E. C.). This work was also supported by grants from the Gerry Foundation and Amyloid Research Foundation (to M. S.), the Young Travel Award (to V. T.-R.), and the Departmental Massachusetts Lions Eye Research Fund.

¹ To whom correspondence should be addressed: Boston University School of Medicine, 80 E. Concord St. L904, Boston, MA 02118. Tel.: 617-638-5099; Fax: 617-638-5339; E-mail: vickery@bu.edu.

² The abbreviations used are: AL, primary amyloid; GAG, glycosaminoglycan; LC, light chain; HS, heparan sulfate; SAA, serum amyloid A; CS, chondroitin sulfate; DS, dermatan sulfate; VL, variable domain; CL, constant domain;

AFM, atomic force microscopy; TEM, transmission electron microscopy; ThT, thioflavin T; OS, *O*-sulfated; A β , amyloid β .

GAGs and LC fibrils may allow the development of drugs to interfere with those interactions and retard LC fibrillogenesis.

In this study, we demonstrate that a post-translationally modified, full-length (containing variable, VL and constant, CL domains), amyloidogenic LC protein (AL-00131), purified from AL patient urine, can form oligomers and fibrils under physiologic conditions (concentration, temperature, and pH) *in vitro*. Our studies focused on $\kappa 1$ proteins because they are from an LC subfamily that is highly represented in our amyloid clinic population (18). Although GAGs are a minor component of the tissue extracts from patients with AL amyloid, we hypothesize that HS plays a critical role in the formation of amyloidogenic oligomers and fibrils. The periodicity of GAGs along the fibrils generated *in vitro* under physiologic conditions was similar to that detected in the *ex vivo* samples. The transitions that occur are promoted by the presence of sulfated GAGs such as HS and may depend on differences in pI between VL and CL regions (19). Both HS and heparin caused enhanced formation when compared with dermatan sulfate (DS) and CS. In addition, the rate and extent of oligomer and fibril formation varied with different post-assembly modifications of GAGs. The response and association of amyloidogenic LC with GAGs are specific and are not generated with a non-amyloidogenic LC protein (MM-96100, derived from the urine of a patient with myeloma and no amyloid disease).

EXPERIMENTAL PROCEDURES

Tissue Amyloid Fibril Extraction—All clinical materials were obtained from a deidentified repository of amyloid patient samples and data maintained with approval of the Boston University Medical Campus Institutional Review Board. *Ex vivo* AL amyloid fibrils were obtained from tissue samples of patients with $\kappa 1$ (AL-98002, AL-03-261) LCs using the classical water extraction method of Skinner *et al.* (20). Fibrils were collected separately as water wash suspended fractions, and the upper layer was pelleted and lyophilized. Fibril extracts were examined for associated GAGs. Atomic force microscopy (AFM) and transmission electron microscopy (TEM) were performed, and measurements on the fibril extracts served as a baseline for *in vitro* experiments.

Light Chain Purification and Preparation—Light chains were purified from urine collected from patients with AL amyloidosis, which featured the overproduction of a monoclonal $\kappa 1$ LC (21). Briefly, urine samples were exhaustively dialyzed against double distilled H₂O, lyophilized, and albumin-depleted using Affi-Gel Blue (Bio-Rad Laboratories). Light chains were isolated by chromatographic separation on a Sephacryl S-200 column (Amersham Biosciences, Buckingham, UK) and characterized by mass spectrometric analyses (19). The amyloidogenic $\kappa 1$ LC (AL-00131) (GenBankTM accession number EF589383) was chosen for analysis because biological responses have been studied, and it is structurally similar to the previously studied amyloidogenic $\kappa 1$ LC, AL98002 (GenBank accession number AF124197) (19, 21, 22). In addition to their highly homologous primary sequences, both amyloidogenic LCs are S-cysteinylylated at Cys-214. A non-amyloidogenic LC (MM-96100) from a patient with multiple myeloma and no amyloid disease was purified from urine and served as a control. For all

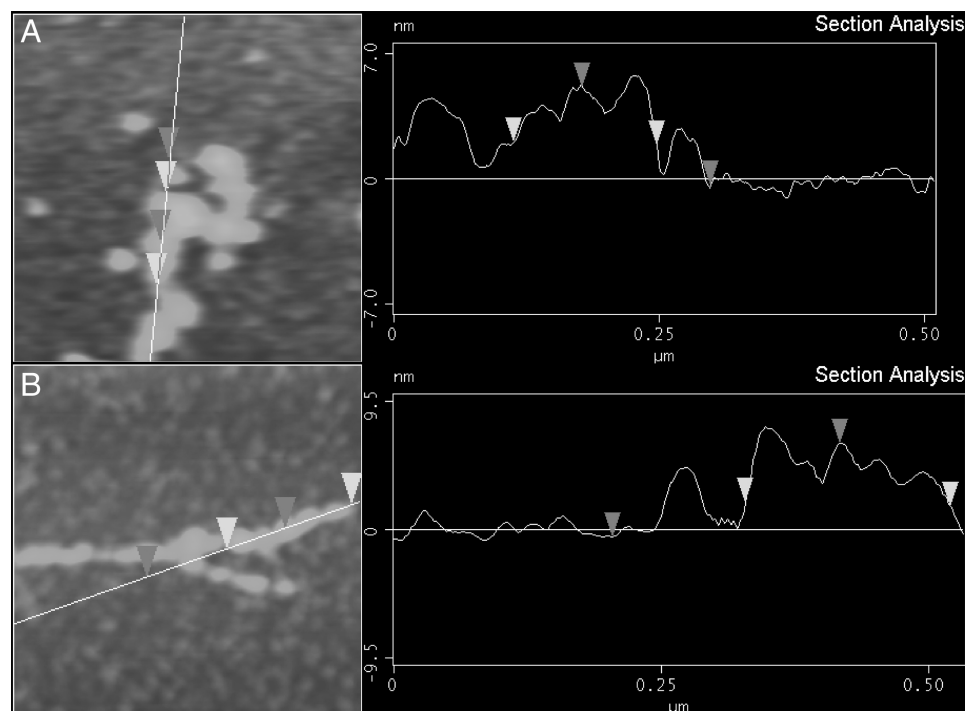
samples, lyophilized preparations were resuspended in appropriate buffer, sonicated, and filtered with 100-kDa spin filters to remove insoluble aggregates.

Glycosaminoglycans—Bovine kidney HS, dextran with a molecular weight of 71,000, dextran with a molecular weight of 500,000, and dextran sulfate were purchased from Sigma. CS and DS were purchased from Seikagaku Corp. (Tokyo, Japan). Heparin, the de-O-sulfated form of heparin, and the oversulfated form of heparin (over-OS-heparin) were purchased from Neoparin Inc. (Alameda, CA). In the oversulfated form of heparin, the primary hydroxyls on glucosamine residues and a large proportion of secondary hydroxyl groups in disaccharide units were substituted by O-sulfate esters. Likewise, the de-O-sulfated heparin is a derivative in which all O-sulfate esters of heparin have been removed without changing the backbone structure with most of the negative charge contributed by O-sulfates eliminated (as described by Neoparin Inc.).

Negative Staining—The morphologies of fibrils extracted from amyloid-laden tissues and those generated *in vitro* from purified urinary LCs were assessed by TEM and AFM. The fibrils generated *in vitro* were analyzed in the presence and absence of GAGs. Briefly, 0.25 mg/ml LC in 10 mM Tris-HCl (pH 7.0) was incubated and applied to 300-mesh carbon-coated copper grids that had been glow-discharged to render the surface hydrophilic (22). The LC was adsorbed onto the grid for 25 min at room temperature, and the excess solvent was removed by wicking with filter paper. Samples deposited on grids were stained negatively with 1% sodium phosphotungstic acid for 10 s. Sodium phosphotungstic acid was removed, and the sample was air-dried. Grids were visualized with a Philips CM12 transmission electron microscope (Philips Electronics, Andover MA). To monitor the structures generated over time, 12 randomly chosen EM areas, 45 × 45 μm each in size, for every sample grid were visualized, and the protofilaments, protofibrils, and fibrils were counted. The structures were divided into the same categories according to fibril width (less than 3 nm, protofilament; greater than 3 nm but less than 7 nm, protofibril; greater than 7 nm, fibril). For those samples with more than 1000 such structures at 2 weeks, the number is listed as >1000 (see Table 2).

Cuprolinic Blue Staining—The dye has been used to demonstrate the presence of sulfated GAG chains along collagen fibrils and is used here to show the association of GAGs with fibrils in tissue, fibrils extracted from tissue, or fibrils generated *in vitro* (23, 24). Tissue fibril extracts were hydrated with a 10 mM Tris-HCl (pH 7.0) buffer overnight and centrifuged. Pellets were embedded in low melt agarose, fixed, and stained with 1% Cuprolinic blue (in buffer containing 0.3 M MgCl₂) for 24 h (23, 24). Fibrils were dehydrated and processed for TEM. Sections were stained with filtered supernatant of saturated solution of uranyl acetate for 40 min and air-dried a distilled H₂O rinse. $\alpha\beta$ fibrils generated *in vitro* and frozen autopsy tissue sample containing amyloid fibrils were used as positive controls (data not shown).

Atomic Force Microscopy—*Ex vivo* fibrils were imaged with a MultiMode atomic force microscope controlled by Nanoscope IIIa electronics (Digital Instruments, Santa Barbara, CA) equipped with a 12- μm scanner (E-scanner). Tapping mode



C

	Height (nm)	Periodicity (nm)	Total GAG (ug/mg fibril extract)
Liver (A)	5.6 ± 0.1	47.5 ± 0.8	9.4
Heart (B)	5.2 ± 0.1	43.1 ± 0.9	14.0

FIGURE 1. Characteristics of amyloid fibrils extracted from tissue. A and B, characteristics of AL κ 1 (AL-98002) protofibrils extracted from liver (A) and heart (B). AFM images are 250 × 250 nm. The line drawn through the image is represented graphically on the right, optimized for periodicity. White arrowheads indicate periodicity measurements, and gray arrowheads indicate height. Multiple measurements were made for these and other fibrils. C, height and periodicity of extracted fibrils (mean ± S.D.) and total GAG in extracted fibrils.

etched silicon probes with 100- μ m-long rectangular cantilevers ($k = 20$ –80 newtons/m) (Veeco Probes) were used. Tips were exposed to UV light prior to use. Images were taken in air using tapping mode. For background noise isolation, the samples and scanner were placed on an air table. Samples were imaged after fibril extract was deposited onto a freshly cleaved mica surface, allowed to set for 3 min at room temperature, and then washed with distilled H₂O and dried under a nitrogen stream.

To examine the role of GAGs and specifically the effect of sulfation on LC oligomer formation and fibrillogenesis, LC samples were incubated in the presence or absence of selected GAGs for 3.25 h (oligomer formation assay) up to 2 weeks (fibril formation assay). Aliquots were imaged wet using a Dimension 3100 atomic force microscope with a 20- μ m scanner (Digital Instruments) (at the Photonics Laboratory, Boston University). Imaging was performed after 3 h or weekly to monitor change in structure. The formation of oligomers was further verified by using an antibody A11 (BioSource Inc., Camarillo, CA) directed against oligomers (25). The structures were divided into categories by height: less than 3 nm, protofilament; greater than 3

nm and less than 7 nm, protofibril; and greater or equal than 7 nm, fibril.

Glycosaminoglycan Analysis—GAGs were determined using the dimethylmethylene blue assay developed by Farndale *et al.* (26). The fibril extracts were digested with proteinase K and papain (11) and subjected to digestion with heparinase I and III (1 and 2 units/ml, respectively, pH 7.0) for 5 h at 37 °C in PBS, chondroitinase AB (0.5 units/ml), pH 8, for 5 h in 50 mM sodium acetate, chondroitinase ABC (1 units/ml), pH 7.3, for 5 h in 50 mM sodium acetate buffer or keratanase II (0.01 unit/ml; pH 5.9).

Thioflavin T Fluorescence—Changes in structure were monitored using ThT (27). Thioflavin T forms a complex with fibrils, and the fluorescence intensity is proportional to the amount of fibrils that are present when the concentration of ThT is held constant (16). The effects of ThT buffer solutions on LC structure were evaluated by circular dichroism (CD) and confirmed by TEM and AFM (27–30).

In the long term ThT assays, 0.25 mg/ml light chain samples were prepared in 10 mM Tris-HCl buffer (pH 7.0) and incubated at 37 °C with a gentle rocking at 25 rpm on a New Brunswick E24 shaker (New Brunswick Scientific, Edison, NJ) for up to 1 week. Aliquots were removed daily, incubated in 10 μ M ThT, and analyzed by measuring fluorescence intensity levels (excitation, 450 nm, and emission, 482 nm) on a PerkinElmer Life Sciences 650S fluorometer (PerkinElmer Life Sciences). A 5-nm slit grating was used for both excitation and emission. Fluorescence data were expressed as arbitrary units after background levels were subtracted from readings at each time point. Buffer and HS alone were assayed at each time point to ensure that HS did not alter fluorescence. ThT assay was performed daily.

Short term ThT assays were performed to provide comparison for other studies. Light chain samples (1 mg/ml) were prepared in 10 mM Tris/NaCl buffer (pH 7.0), incubated at 60 °C, and subjected to rocking at 250 rpm. Aliquots were taken at intervals ranging from 0 to 72 h, incubated in 10 μ M ThT, and analyzed as described above. Appropriate controls were included.

RESULTS

Ex Vivo Amyloid Tissue Proto Fibrils—Structural characteristics of the protofibrils obtained from the AL-98002 tissue

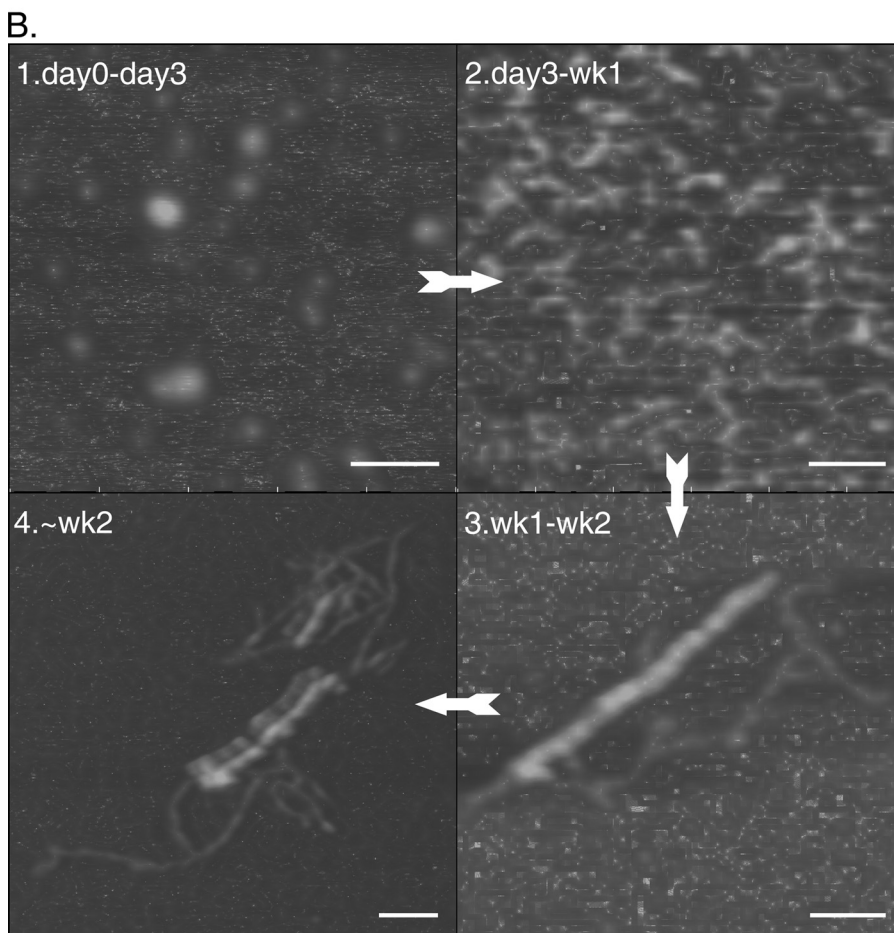
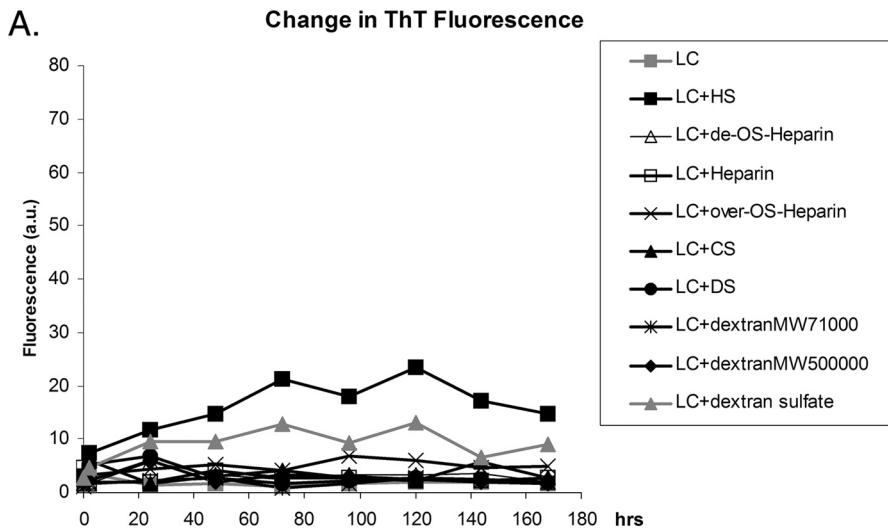


FIGURE 2. Long term AFM and ThT fluorescence study demonstrates role of GAGs. *A*, urinary LC AL-00131 purified from urine (0.25 mg/ml) was incubated in the presence or absence of GAGs in Tris-HCl (pH 7) at 37 °C and 25 rpm for 200 h, and ThT fluorescence was measured over time. The experiment was repeated three times with comparable results. *a.u.*, arbitrary units; *MW*, molecular weight. *B*, AFM of changes of urinary LC AL-00131 after 2 weeks of incubation. The LC oligomers (1) undergo a structural rearrangement and (2) aggregate to form protofilaments (3). The protofilaments can intertwine to form protofibrils (4). Protofibrils can either intertwine or fold to form fibrils. Each of these experiments was repeated more than 20 times. Scale bar = 100 nm.

extracts were determined using AFM (Fig. 1). The mean heights for liver and heart protofibrils were 5.2 and 5.6 nm, respectively; the periodicity for liver and heart protofibrils ranged from 43 to 47 nm, respectively (Fig. 1). The range is thought to be due to

population variability within each tissue and may represent fragmentation (31). Sulfated GAGs associated with amyloid fibrils were determined after treatment with proteinase K and papain and measured using dimethylmethylene blue assay (525 nm). Extracts were further examined after polysaccharide lyase digestion (including chondroitinase ABC, chondroitinase AC, heparinase I, heparinase III, and keratanase). The total GAG was 9.4 and 14 $\mu\text{g}/\text{mg}$ of dry weight of fibril extract for liver and heart, respectively (Fig. 1). In addition, HS ranged from 3 to 9 $\mu\text{g}/\text{mg}$ of dry weight of fibril for the $\kappa 1$ tissue, which represented 79% of GAGs in liver, 83% of GAGs in kidney, and 93% of GAGs in spleen. Chondroitin sulfate ranged from not detectable to 0.9 $\mu\text{g}/\text{mg}$ of dry weight, and keratan sulfate was not detected (data not shown). Although these results demonstrated that GAGs were present in the fibrillar fractions, it did not indicate how the GAGs were associated with the fibrils.

Role of GAGs and Sulfation on LC Amyloid Formation—ThT assays were performed to assess the role of GAGs on the formation of crossed β -sheet structure and amyloid fibrils with LCs. We examined the change in fluorescence over a 1-week period, with aliquots analyzed daily (Fig. 2A). Moderate conditions were used to incubate LC and GAGs: physiologic concentration of LC (0.25 mg/ml), 37 °C, 10 mM Tris-HCl (pH 7.0), and 25-rpm rotation, which was applied over the time period. AFM studies were performed in parallel, and structure and ThT fluorescence were assessed. Aggregation was not detected in the long term studies (data not shown).

Fluorescence of LC alone was negligible. Heparan sulfate (0.5 mg/ml) enhanced ThT fluorescence within the first 24 h and remained elevated (Fig. 2A). Incubation with an equivalent concentration of CS or DS did not enhance fluorescence when compared with LC alone (Fig. 2A). Furthermore, samples containing either GAGs alone or buffer alone showed no increase in fluorescence

Role of GAGs in AL Fibrils

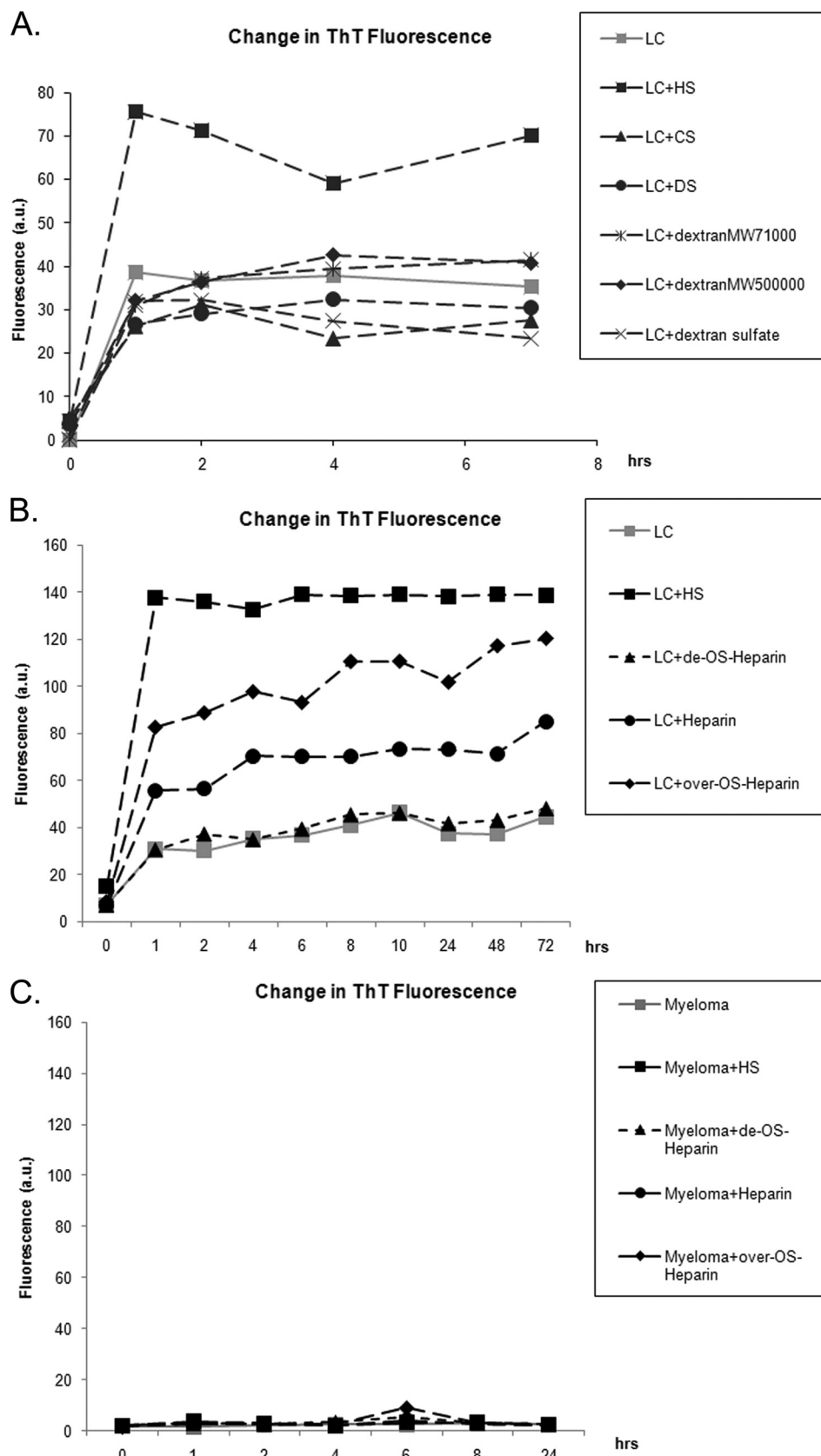


FIGURE 3. Sulfated GAGs alter ThT fluorescence of urinary LC AL-00131 in short term studies. A, LC (1 mg/ml) incubated in the presence or absence of GAGs in Tris-HCl (pH 7) at 60 °C and 250 rpm (ThT: excitation, 450 nm, emission, 482 nm). MW, molecular weight. B, LC (1 mg/ml) incubated in the presence or absence of HS, heparin, and heparin derivatives. C, myeloma LC (MM96100) incubated in the presence or absence of different GAGs under the same conditions. Experiments were repeated three times and gave similar results.

over background (data not shown). Dextran of two different molecular weights, 71,000 or 500,000, and dextran sulfate were used as controls. Although neither molecular weight of dextran generated an enhanced signal, dextran sulfate did generate an increase over background. However, the fluorescence generated by LC and dextran sulfate was less than that generated by LC and HS (Fig. 2A). These data indicate that HS may play a unique role on amyloid fibril formation. In addition, experiments were conducted using the recombinant AL-00131 protein (lacking post-translational modifications), and the fibril formation rate was quite different (data not shown).

Although ThT fluorescence of LC alone was not detected above control in long term studies conducted at physiologic concentrations, protofilaments, protofibrils, and fibrils were detected when the same experiments were analyzed using AFM (Fig. 2B). The lack of detectable fluorescence in LC alone samples may be due to the sparseness of the structures that we did detect in the multiple AFM images analyzed. This may explain the lack of detection in previous studies (28). These indicate that multiple forms of analysis are important and that ThT measurements may be a less sensitive technique requiring a larger population of LC to form fibrils. To compare our data with that of others, a number of experiments were performed where we accelerated the process using a high concentration of AL-00131 (1.0 mg/ml) incubated in 10 mM Tris-HCl buffer (pH 7.0) at 60 °C and subjected to 250 rpm to compare the interaction of LC with GAGs. These were conducted in the presence or absence of GAGs, and ThT fluorescence was analyzed at hourly intervals. When LC was incubated by itself, there was an increase in fluorescence in the first hour of incubation that was followed by a plateau. The presence of CS or DS did not enhance fluorescence when compared with LC alone. Because both

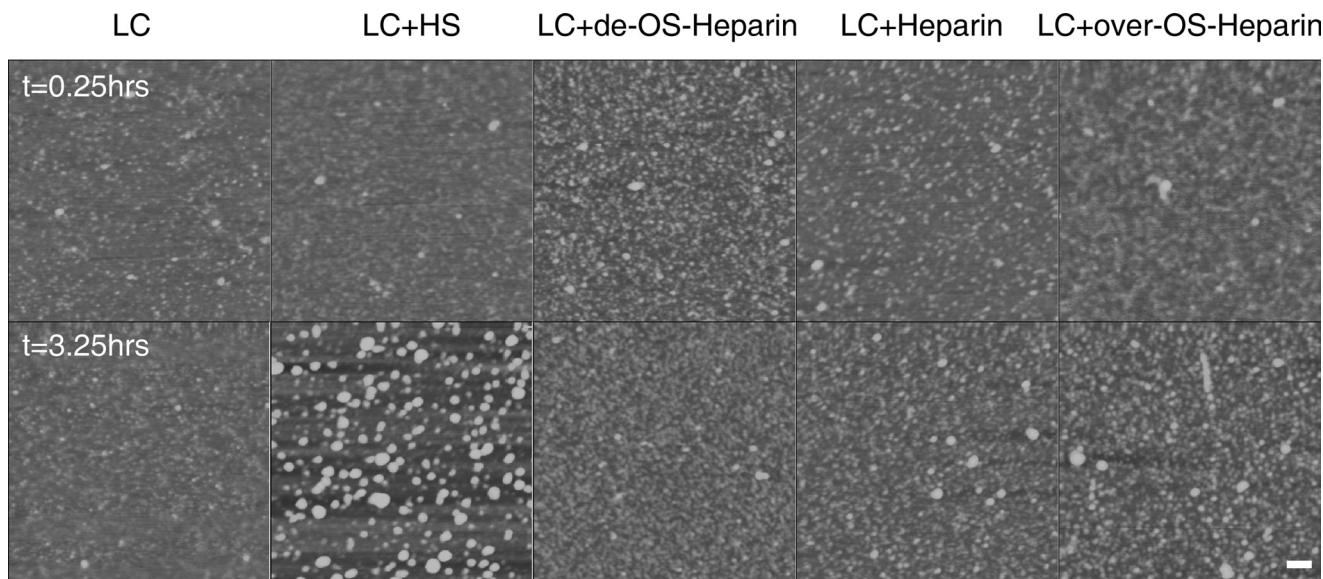


FIGURE 4. **Morphology of oligomers is altered by HS.** LC AL-00131 was incubated in the presence or absence of GAGs using AFM ($1 \times 1 \mu\text{m}$). These experiments were repeated three times and gave similar results. Scale bar = 100 nm.

dextran and dextran sulfate have been shown to increase the ThT signal when added to LC in other systems, we repeated the experiments under these harsher conditions (33). Our data showed that dextran caused a slight increase in ThT when compared with LC alone, and a maximum increase was consistently seen with the addition of HS (Fig. 3, A and B). Experiments performed with buffer or GAG alone did not cause an increase in fluorescence over time (data not shown). These data also suggest that HS may play a unique role on amyloid fibril formation when compared with other GAGs.

Similar experiments were performed on heparin derivatives, and the results were compared with heparin and HS. When the LC was incubated by itself or in the presence of the de-OS-heparin, there was an initial minor increase in fluorescence followed by a plateau. The addition of over-OS-heparin resulted in a large initial increase in fluorescence followed by a gradual increase over time. A gradual increase was also observed with the addition of heparin. The response was predictably intermediate between those produced by de-OS-heparin or over-OS-heparin. The addition of HS to LC resulted in a rapid increase in fluorescence in the first hour that was 6-fold greater than LC alone and 2-fold greater than LC and heparin. The rapid increase in ThT signal was followed by a plateau (Fig. 3B). These data indicate that both the GAG species and their sulfation are important mediators. In addition, when experiments were performed in parallel with the urinary non-amyloidogenic LC protein (MM-96100) with HS, heparin, de-OS-heparin, or over-OS-heparin, there was no increase in fluorescence (Fig. 3C). These data indicate that the interaction of amyloidogenic LC proteins with GAGs is distinct from that of non-amyloidogenic LC proteins. Together, these data indicate that HS causes the most rapid and sustained response of GAGs that were tested and that the response of amyloidogenic LCs is distinct from that of non-amyloidogenic LCs.

Oligomer Formation Is Mediated by Sulfation of Glycosaminoglycans—Oligomer formation experiments were performed with the $\kappa 1$ LC, AL-00131, purified from urine. The LC

(0.25 mg/ml) was suspended in the presence or absence of GAGs at 37°C in 20 mM Tris, 30 mM NaCl buffer (pH 7.0) and rocked gently at 25 rpm. For each LC solution, oligomer formation was examined immediately using AFM, after deposition of the solution on a freshly cleaved piece of mica and again after 3 h of incubation at 37°C with gentle rocking (25 rpm) (Fig. 4). Verification of oligomers was performed using an immunoblot assay at 3 h with an antibody directed against oligomers that does not recognize aggregates (data not shown).

The ability of LC to form oligomers was compared in solutions that contained LC and one of the following: HS, heparin, and the modified forms of heparin (the de-O-sulfated form of heparin or over-OS-heparin) (Fig. 4). In the presence of LC, there was a negligible change in oligomer height and size distribution over time. However, oligomer formation was enhanced in the presence of heparin and HS. When LC was incubated in the presence of the de-OS-heparin, there were only minor changes in gross morphology up to 3 h, whereas the over-OS-heparin showed a dramatic change in morphological appearance (Fig. 4).

Depth analysis was performed on six randomly chosen 500×500 -nm grid areas for each sample at 0.25 and 3.25 h using Nanoscope Version 5.31r1. Depth analysis provides depth comparisons between two dominant features: the mica background and oligomers, and the depth at maximum, which is the depth at the maximum peak on the histogram. The data did not change over time when LC was incubated alone or in the presence of de-OS-heparin (constant at ~ 2.0 – 3.5 nm) but increased 2-fold when LC was incubated in the presence of either over-sulfated heparin (ranging from ~ 1.5 – 3.0 to ~ 3.3 – 6.3 nm) or HS (ranging from ~ 4.8 – 6.5 to ~ 7.5 – 12.6 nm) (Fig. 5A).

Roughness analysis was performed to determine the surface of the oligomers. Prior to analysis, the image was flattened, and a similar region of exactly the same size was analyzed for each sample (Nanoscope Software). When the LC was incubated

Role of GAGs in AL Fibrils

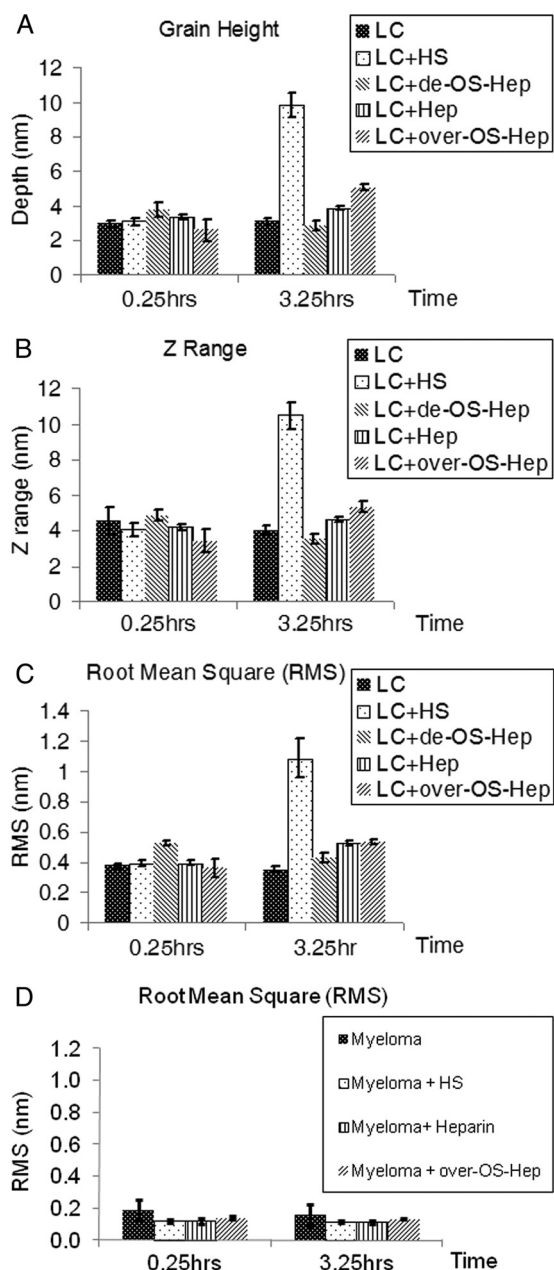


FIGURE 5. AFM analysis of oligomers. AL-00131 was incubated in the presence or absence of GAGs. *A*, average grain height at maximum. *Hep*, heparin. *B*, average Z range. *C*, average root mean square (RMS) of AL-00131, with the presence or absence of GAGs, changing over time. *D*, average root mean square of myeloma MM96100, with the presence or absence of GAGs, changing over time. Experiments were repeated three times and gave similar results.

with a sulfated GAG, there was a 2–3-fold increase in Z range, which is defined as the peak-to-valley difference in height. However, there was only a minor increase for both the LC alone and the LC incubated in the presence of de-OS-heparin (Fig. 5*B*). Furthermore, when GAGs were incubated alone, there was no change over time (Fig. 5*B*). If an increase in the oligomers occurred over time, we hypothesized that there would be an increase in root mean square (RMS), which is defined as the standard deviation of the Z values within the defined region. In fact, there was a 2-fold increase detected when the LC was incubated in the presence of sulfated GAG. In contrast, when the LC

was incubated alone or in the presence of the de-OS-heparin, only a negligible increase in Z values was detected (Fig. 5*C*). When parallel experiments were performed with the non-amyloidogenic LC protein, MM-96100 in the presence of GAGs, there was no significant change detected (Fig. 5*D*).

Formation of Protofibrils and Fibrils Are Mediated by GAGs—To determine the role of sulfated GAGs on fibril formation and morphology, experiments were conducted under physiological conditions (10 mM Tris, 30 mM NaCl, pH 7.0; 37 °C) (Fig. 6). The κ 1 LC (AL-00131) protein (0.25 mg/ml) was incubated alone or in combination with 0.5 mg/ml HS, heparin, over-OS-heparin, or de-OS-heparin, and the products were analyzed using AFM. After 1 week of incubation, protofibrils were detected only in the samples containing either HS or the over-OS-heparin. After 2 weeks of incubation, protofilaments/protofibrils were detected in all samples. Although protofibrils formed an extensive matrix in the sample with HS, there was only a sparse number in the LC alone sample or in those with de-OS-heparin or heparin (Fig. 6). At 2 weeks, protofibrils were prominent in the over-OS-heparin samples and displayed a uniform matrix along the mica surface. The heights and periodicities of the protofilaments, protofibrils, and fibrils were measured (Figs. 4 and 5). Atomic force microscopic images of LC alone at 14 days detected protofilaments ranging in height from 1.5 to 3 nm and a periodicity of 40 nm, whereas the protofibrils had a mean height of 5 nm and a periodicity of 40 nm (Table 1). When LC and HS were examined, the mean height of the protofilaments was 1.7 nm with a periodicity of 30–40 nm, and the protofibrils had a height and periodicity of 5 and 45 nm, respectively (Table 1). There were no significant differences in the dimensions for protofilaments, protofibrils, or fibrils detected among any of the samples. The results were in contrast to the substantial differences observed in the LC oligomers.

Although AFM provides high resolution, the area that is analyzed is limited for population analysis. Therefore, negative staining TEM was used to examine a wider population of samples. No significant differences were observed in the morphology of the fibrils when the LC was incubated in the presence of HS. However, the samples incubated in the presence of HS had a 500-fold increase in fibril cluster (defined as the total number of protofilaments, filaments and fibrils) formed over a 1-week period when compared with other samples (Table 2). Both heparin and over-OS-heparin enhanced the formation of fibril clusters, with a 10–40-fold increase. In contrast, the presence of de-OS-heparin did not alter the fibril cluster amount when compared with LC alone (Table 2). By 2 weeks, the number of fibril clusters exceeded 1000 in the presence of HS or the over-sulfated form of heparin. The amount of LC was kept constant in all of the conditions.

Interaction of GAGs along Fibrils—To determine whether GAGs were associated with the fibrils of the κ 1 LC, fibrils were embedded in agarose, incubated with Cuproline blue, which binds uronic acid residues, and examined. Electron-dense filaments representing GAGs were detected in a periodic pattern along the fibrils (Fig. 7). The low contrast in the image is due to the concentration of uranyl acetate necessary for Cuproline blue staining. The periodicity of GAGs found along the κ 1 LC

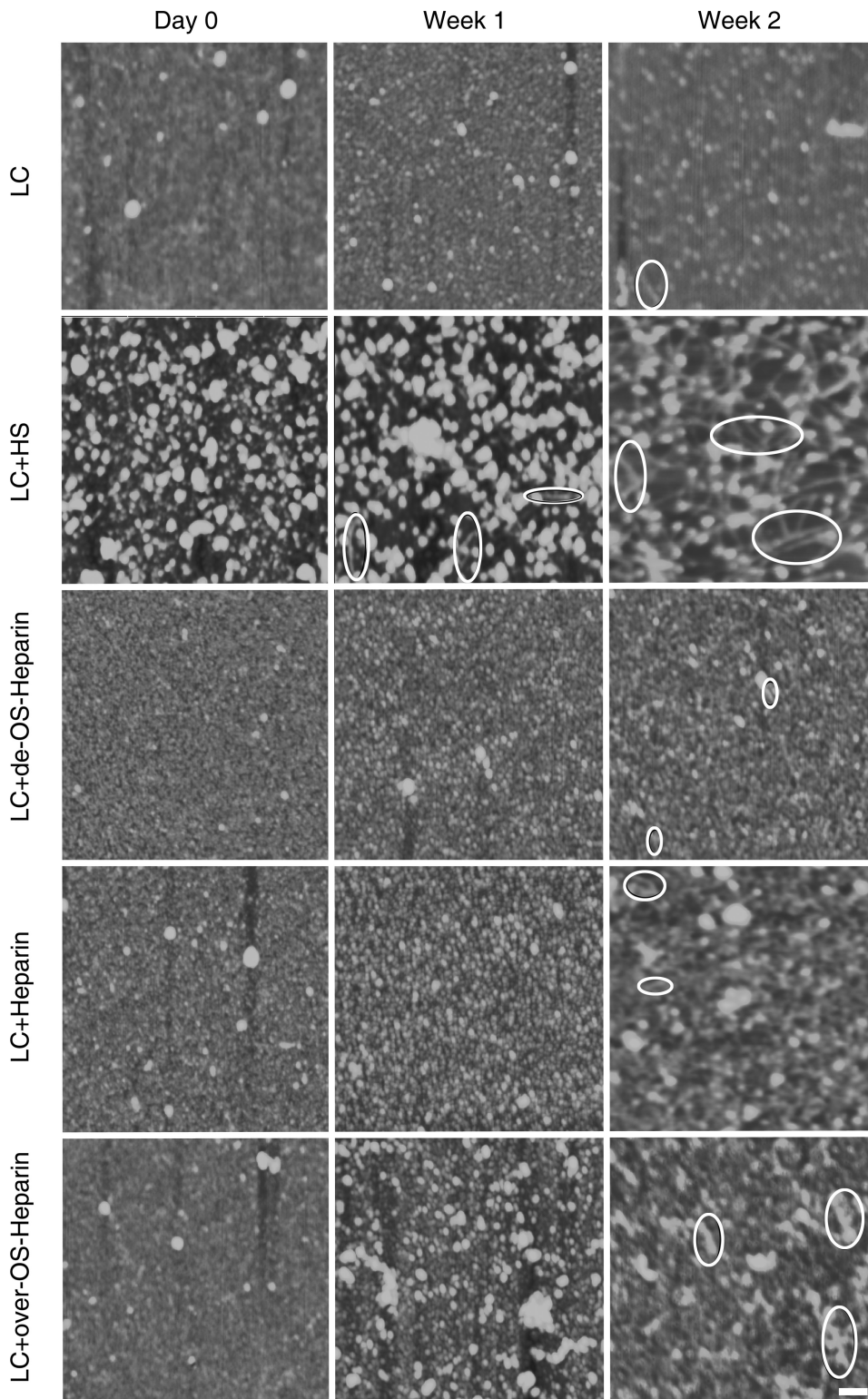


FIGURE 6. Long term analysis of change in structure in the presence or absence of GAGs. AFM images of LCs in the presence or absence of GAGs over 2 weeks ($1 \times 1 \mu\text{m}$). The circles indicate examples of the protofilaments and protofibrils formed over time. The structures were only detected in the presence of HS at 1 week. Experiments were repeated more than 20 times, with similar results. Scale bar = 100 nm.

fibril was 0.48 GAGs/nm. The dimension of fibrils was not altered by Cuprolic blue dye binding, and comparisons were made to negatively stained fibrils at an equivalent magnification (Fig. 7A). A number of controls were tested to demonstrate

the specificity of Cuprolic blue staining. After treatment with heparinase, no structures were detected above background (Fig. 7C). Electron-dense filaments were prominent in the AL-98002 tissue (Fig. 7D, arrowheads), and these were greatly reduced after digestion with heparinase (Fig. 7E). In proof of principle experiments, amyloid fibrils were formed by incubating $A\beta_{1-40}$ in the presence or absence of HS and stained with Cuprolic blue. The $A\beta_{1-40}$ fibrils formed in the absence of sulfated GAGs were not detected when compared with images incubated with HS (data not shown). These data indicate that HS can be detected along fibrils, and the periodicity suggests the presence of a high level of organization.

We then investigated whether the HS chains associated with fibrils found in the *ex vivo* samples were similar to fibrils cultured *in vitro*. AL-00131 was incubated under physiologic concentration in the presence of HS, and the samples were stained with Cuprolic blue and imaged using electron microscopy. Electron-dense filaments were detected along the LC fibril, and the GAGs had a periodicity of 0.42 GAGs/nm (Fig. 8). The similarity in the periodicity of GAG chains along the *ex vivo* fibrils (Fig. 8) and those LC fibrils formed *in vitro* was striking. Additional control experiments conducted with the urinary non-amyloidogenic LC, MM-96100, and GAGs did not produce amyloid fibrils, and no electron-dense filaments were present (data not shown). In addition, oligomer formation was not obvious (data not shown), and ThT fluorescence signal was negligible (Fig. 3C). Taken together, the results indicate that amyloid fibrils formed *in vitro* under moderate conditions with amyloidogenic urinary LCs are very similar to

ex vivo fibrils extracted from tissue deposits, and the rate and the overall morphology are significantly influenced by the presence of specific polysaccharide chains on GAGs.

TABLE 1

Effect of GAGs on the fibrils generated *in vitro*

Height and periodicity of protofilaments/protofibrils/fibrils formed in AL-00131 +/- GAGs were measured after 2 weeks of incubation.

Height	LC	LC + HS	LC + desulfated heparin	LC + heparin	LC + OS-heparin
<i>nm</i>					
Protofilaments	1.5–3	1.5–2.0	1.0–1.5	1.5–3	1.5–2.0
Protofibrils	4.5–6.0	4.5–6.0	2.0–2.2	3.5–4.5	4.5–6.5
Fibrils	7.5–10.0	7.0–8.0	7.0–7.2	7.0–10.0	7.0–10.0
Periodicity	LC	LC + HS	LC + desulfated heparin	LC + heparin	LC + OS-heparin
<i>nm</i>					
Protofilaments	~40	30–40	20–40	~40	~40–50
Protofibrils	~40	40–50	30–40	~80	~40–50
Fibrils	90–100	40–50	~90	80–100	~80

TABLE 2

Effect of GAGs on the formation of fibrils generated *in vitro*

Protofilaments/protofibrils/fibrils (defined as fibril clusters) were detected at three time points when LC AL-00131 was incubated in the presence or absence of GAGs. The fibril cluster was counted in 12 45 × 45-μm areas on EM grids. Numbers over 1000 were not counted and noted as >1000. Representative images of week 1 AL-00131 in the presence or absence of HS are included.

Fibril clusters/540 μm ²	Day 0	Week 1	Week 2
AL-00131	3.8 ± 1.6	19.4 ± 3.4	342.2 ± 69.3
AL-00131 + HS	1.3 ± 1.1	939.7 ± 106.5	>1000
AL-00131 + de-OS-Heparin	1.3 ± 1.4	16.8 ± 7.7	331.8 ± 64.5
AL-00131 + heparin	1.7 ± 1.5	22.1 ± 7.6	672.8 ± 51.3
AL-00131 + over-OS-heparin	1.7 ± 1.2	68.0 ± 10.1	>1000

DISCUSSION

The κ1 LCs used in the present study have previously been comprehensively characterized by mass spectrometric analyses and examined in cardiac cell cultures for their effects on cellular responses (19, 22). Although other studies have examined LC structure and the effect of GAGs on fibril formation, most were performed with recombinant LCs that contained only the VL domain and did not possess post-translational modifications (27–29). The present study indicates that the specific post-translational modifications present within full-length LC dramatically influence the effects of HS on fibril formation. The importance of the presence of both the VL and the CL regions for aggregation and stability has been recently proposed (34, 35) and suggests that there may be a difference in the binding of sulfated GAGs. The latter reports support the demonstrated differences in pI detected within LCs, specifically with the VL region tending to be more acidic (18). Our preliminary studies using recombinant protein lacking the post-translational modifications resulted in a different rate and size (data not shown), indicating the importance of the modifications. We hypothesize that the modifications, present throughout the LC, create distinct regions that provide unique interaction sites that may be essential to the process of fibrillogenesis. One of the goals of this study was to determine how HS, along with heparin and its derivatives, alters the formation of oligomers and fibrils in long term studies. In addition, we sought to characterize the presence of GAGs along the fibrils. Atomic force microscopy revealed that HS accelerated the rate of LC oligomer formation. Although the oversulfated form of heparin is more sulfated than HS, the latter consistently elicited the highest ThT signal and most rapid transition from oligomer to protofibril and fibrillar conformation. In contrast, the desulfated forms of heparin formed structures that resembled LC alone. Thus, the activity of HS is not simply correlated to the level of sulfation

but more likely reflects chemically specific interactions between motifs within HS and properly modified AL LCs.

Cuproinic blue staining of sulfated GAG along the fibrils in tissue and in the *ex vivo* preparations was detected. There were strikingly similar periodicities in fibrils formed *in vitro* and those from *ex vivo* preparations. Previously, Cuproinic blue or sulfated Alcian blue have been used to show the association of sulfated GAGs with collagen fibrils (23, 24). Our images on tissue confirm those studies performed previously with Cuproinic blue or sulfated Alcian blue on tissue with amyloid-associated amyloid (36, 37). In other reports, Jiang *et al.* (38) showed that the interaction of heparin and AL LC depended on concentration and mass. The *in vitro* kinetic studies suggested that the electrostatic forces were important in the interactions between LC and GAG. In addition, apoSAA has been shown to contain specific binding sites for heparin and HS but not for other GAGs (9). Furthermore, Elimova *et al.* (39) proposed that there is a histidine-dependent pH-sensitive HS-binding site in SAA. Their data suggest that the environment may become more hypoxic, changing the charge on the histidine and thus facilitating HS-dependent aggregation.

The fibrillogenesis that we detected in our AFM studies on LC alone (Fig. 2) is similar to that proposed by Zhu *et al.* (27). Our studies were performed using long term and short term studies. To simulate the more gradual progression of disease, long term studies (up to 2 weeks) were performed at 37 °C and pH 7.0 using a physiologic concentration of LC. In contrast, the short term experiments were performed under conditions of high concentration of LC, with rapid shaking (250 rpm), and at high temperature (60 °C). Although it is not surprising that the AFM showed a heterogeneous population in the long term experiments, we believe that this reflects the more normal evolution of the fibrils, as supported by our AFM data from the *ex vivo* fibril extracts. In both sets of studies, we found that the addition of the desulfated heparin did not alter the values obtained with LC alone, whereas HS resulted in a rapid increase in fluorescence. In the long term studies, the HS enhanced oligomer formation and transition to protofibril formation. Although the over-OS-heparin resulted in a rapid increase in fluorescence, it lagged behind the HS in transition to protofibrillar formation. Interestingly, in both short term and long term studies, neither DS nor CS induced ThT fluorescence over background. In addition, the resulting structure from the addition of CS possessed large loose and amorphous fibrils. The changes appear to be specific to amyloidogenic LCs as no

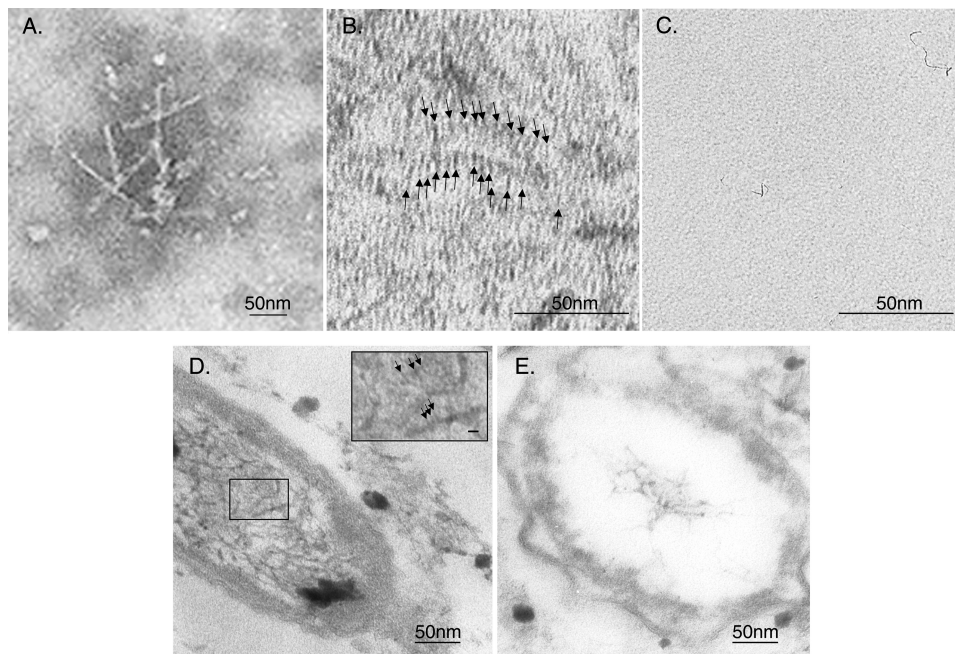


FIGURE 7. Sulfated GAG chains decorate *ex vivo* amyloid fibrils. *A*, negatively stained AL κ 1 protofibrils (ap03-261) extracted from heart and imaged with TEM. *B*, Cuprolinic blue-stained protofibrils extracted from heart (arrows). *C*, Cuprolinic blue-stained protofibrils extracted from heart after heparinase digestion. *D*, Cuprolinic blue-stained AL- κ 98002 liver (arrows). The inset shows stained filaments along the AL fibrils (scale bar = 10 nm). *E*, Cuprolinic blue-stained AL- κ 98002 after heparinase digestion.

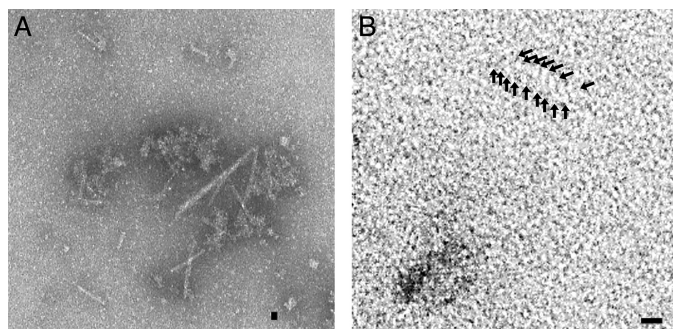


FIGURE 8. Sulfated GAGs are detected along amyloid fibrils generated *in vitro* with HS. *A*, negatively stained AL κ 1 protofibrils AL-00131 generated *in vitro* from urinary extracted LC protein. *B*, Cuprolinic blue (arrows)-stained AL κ 1 protofibrils AL-00131 generated *in vitro*. *C*, negative control image of AL-00131 incubated in the absence of GAGs and stained with Cuprolinic blue. Scale bar = 10 nm.

changes were detected when the GAGs were incubated with non-amyloidogenic LC. At the current time, it is not clear whether the different preparations represent stages in development or whether they are a collection of structures without a linear relationship. Without the ability to tag molecules and monitor the formation over time using methods that do not modify the process, we cannot rule out that other hypotheses are plausible.

Recently, Motamedi-Shad *et al.* (32) proposed that HS induces a new phase, which contains oligomers possessing a β -structure that are capable of forming fibrils. Our data support this hypothesis in the full-length LC protein and suggest that it is the β -structure of the oligomers in the presence of HS that produces the ThT fluorescence. When the role of HS was compared with heparin and its derivatives, the lack of sulfation of the de-OS-heparin resulted in the formation of fibrillar struc-

tures at a rate and manner similar to LC alone. In contrast, heparin and the oversulfated form of heparin showed enhanced fluorescence. These results are supported by the fibril data. Moreover, preliminary work on the structure of HS associated with extracted fibrils from LCs (of known post-translational modifications) indicates a predominance of specific disaccharides. These findings indicate that the effect of proteoglycans on LCs occurs via the interaction of the GAG chain with the amyloid protein. Furthermore, recent work has demonstrated that the most highly sulfated region of HS is the external end (7). We speculate that this region of HS interacts with the amyloid fibrils and plays a role in spatial configuration of the filaments (data not shown).

The increase in ThT fluorescence and fibril formation with the addition of HS is in agreement with solid phase immunobinding assays in which κ 1 LC association with HS has been reported to be 3-fold greater than observed when the GAG was either keratan sulfate or CS (17). Although these earlier studies did indicate that specific GAGs play a role in fibrillogenesis, our studies with Cuprolinic blue on *ex vivo* fibril extracts and fibrils generated *in vitro* demonstrate that the GAG chains interdigitate along the fibril. Our results indicate that the GAG chains promote the wrapping of the fibrils. Furthermore, the long term incubations of this κ 1 LC (AL-00131) under physiologic conditions generated *in vitro* fibrils that had similar dimensions to the fibrils extracted from the tissue, and the periodicity of the GAG chains was similar. When the LC was incubated in the presence of GAGs, there was an alteration in oligomer formation followed by changes in ThT fluorescence and protofibril formation that depended not only on the specific polysaccharide chain, but also on the degree of sulfation.

In AL amyloidosis, the association of GAG with LC protein may depend on a number of factors including post-translational modifications and the difference in pI between VL and CL regions, both of which can alter the binding of GAGs. Future studies will evaluate the interaction of HS with the CL region and address whether specific binding regions could provide a new target site for therapeutics. Our data indicate that the degree and organization of sulfation of the GAG chain may be a critical factor in the initiation of oligomer formation and progression to fibrillogenesis.

REFERENCES

- Chiti, F., and Dobson, C. M. (2006) *Ann. Rev. Biochem.* **75**, 333–366
- Falk, R. H., Comenzo, R. L., and Skinner, M. (1997) *N. Engl. J. Med.* **337**, 898–909
- Souillac, P. O., Uversky, V. N., and Fink, A. L. (2003) *Biochemistry* **42**, 8094–8104

4. Khurana, R., Ionescu-Zanetti, C., Pope, M., Li, J., Nielson, L., Ramirez-Alvarado, M., Regan, L., Fink, A. L., and Carter, S. A. (2003) *Biophys. J.* **85**, 1135–1144
5. Ionescu-Zanetti, C., Khurana, R., Gillespie, J. R., Petrick, J. S., Trabachino, L. C., Minert, L. J., Carter, S. A., and Fink, A. L. (1999) *Proc. Natl. Acad. Sci. U.S.A.* **96**, 13175–13179
6. Trinkaus-Randall, V., Lanza, R., Langer, R., and Chick, E. (2000) *Cornea: Biological Responses*, 2nd Ed., pp. 383–398, Academic Press, Millbrae, CA
7. Staples, G. O., Shi, X., and Zaia, J. (2010) *J. Biol. Chem.* **285**, 18336–18343
8. Stevens, F. J., and Kisilevsky, R. (2000) *Cell. Mol. Life Sci.* **57**, 441–449
9. Ancsin, J. B. (2003) *Amyloid* **10**, 67–79
10. Ohishi, H., Skinner, M., Sato-Araki, N., Okuyama, T., Gejyo, F., Kimura, A., Cohen, A. S., and Schmid, K. (1990) *Clin. Chem.* **36**, 88–91
11. Nelson, S. R., Lyon, M., Gallagher, J. T., Johnson, E. A., and Pepys, M. B. (1991) *Biochem. J.* **275**, 67–73
12. Ailles, L., Kisilevsky, R., and Young, I. D. (1993) *Lab. Invest.* **69**, 443–448
13. Snow, A. D., Kisilevsky, R., Stephens, C., and Anastassiades, T. (1987) *Lab. Invest.* **56**, 665–675
14. Castillo, G. M., Cummings, J. A., Yang, W., Judge, M. E., Sheardown, M. J., Rimvall, K., Hansen, J. B., and Snow, A. D. (1998) *Diabetes* **47**, 612–620
15. Gupta-Bansal, R., Fredericks, R. C., and Brunden, K. R. (1995) *J. Biol. Chem.* **270**, 18666–18671
16. Cohlberg, J. A., Li, J., Uversky, V. N., and Fink, A. L. (2002) *Biochemistry* **41**, 1502–1511
17. Trinkaus-Randall, V., Walsh, M. T., Steeves, S., Monis, G., Connors, L. H., and Skinner, M. (2005) *Am. J. Pathol.* **166**, 197–208
18. Connors, L. H., Jiang, Y., Budnik, M., Théberge, R., Prokaeva, T., Bodi, K. L., Seldin, D. C., Costello, C. E., and Skinner, M. (2007) *Biochemistry* **46**, 14259–14271
19. Prokaeva, T., Spencer, B., Kaut, M., Ozonoff, A., Doros, G., Connors, L. H., Skinner, M., and Seldin, D. C. (2007) *Arthritis. Rheum.* **56**, 3858–3868
20. Skinner, M., Shirahama, T., Cohen, A. S., and Deal, C. L. (1982) *Prep. Biochem.* **12**, 461–476
21. Lim, A., Wally, J., Walsh, M. T., Skinner, M., and Costello, C. E. (2001) *Anal. Biochem.* **295**, 45–56
22. Monis, G. F., Schultz, C., Ren, R., Eberhard, J., Costello, C., Connors, L., Skinner, M., and Trinkaus-Randall, V. (2006) *Am. J. Pathol.* **169**, 1939–1952
23. Scott, J. E., and Haigh, M. (1988) *Biochem. J.* **253**, 607–610
24. Gong, H., Freddo, T. F., and Johnson, M. (1992) *Exp. Eye Res.* **55**, 691–709
25. Kaye, R., Sokolov, Y., Edmonds, B., McIntire, T. M., Milton, S. C., Hall, J. E., and Glabe, C. G. (2004) *J. Biol. Chem.* **279**, 46363–46366
26. Fardale, R. W., Buttle, D. J., and Barrett, A. J. (1986) *Biochim. Biophys. Acta.* **883**, 173–177
27. Zhu, M., Souillac, P. O., Ionescu-Zanetti, C., Carter, S. A., and Fink, A. L. (2002) *J. Biol. Chem.* **277**, 50914–50922
28. McLaughlin, R. W., De Stigter, J. K., Sikkink, L. A., Baden, E. M., and Ramirez-Alvarado, M. (2006) *Protein Sci.* **15**, 1710–1722
29. Suk, J. Y., Zhang, F., Balch, W. E., Linhardt, R. J., and Kelly, J. W. (2006) *Biochemistry* **45**, 2234–2242
30. Yamamoto, S., Yamaguchi, I., Hasegawa, K., Tsutsumi, S., Goto, Y., Gejyo, F., and Naiki, H. (2004) *J. Am. Soc. Nephrol.* **15**, 126–133
31. Enqvist, S., Sletten, K., and Westermark, P. (2009) *J. Pathol.* **219**, 473–480
32. Motamedi-Shad, N., Monsellier, E., Torrassa, S., Relini, A., and Chiti, F. (2009) *J. Biol. Chem.* **284**, 29921–29934
33. Qin, Z., Hu, D., Zhu, M., and Fink, A. L. (2007) *Biochemistry* **46**, 3521–3531
34. Lavatelli, F., Perlman, D. H., Spencer, B., Prokaeva, T., McComb, M. E., Théberge, R., Connors, L. H., Bellotti, V., Seldin, D. C., Merlini, G., Skinner, M., and Costello, C. E. (2008) *Mol. Cell. Proteomics* **7**, 1570–1583
35. Yamamoto, K., Yagi, H., Lee, Y. H., Kardos, J., Hagihara, Y., Naiki, H., and Goto, Y. (2010) *FEBS Lett.* **584**, 3348–3353
36. Gruys, E., Ultee, A., and Upragarin, N. (2006) *Amyloid* **13**, 13–19
37. Snow, A. D., and Kisilevsky, R. (1985) *Lab. Invest.* **53**, 37–44
38. Jiang, X., Myatt, E., Lykos, P., and Stevens, F. J. (1997) *Biochemistry* **36**, 13187–13194
39. Elimova, E., Kisilevsky, R., and Ancsin, J. B. (2009) *FASEB J.* **23**, 3436–3448