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Interaction Analyses of Amyloid β Peptide (1–40) with Glycosaminoglycan Model Polymers

Yoshiko Miura*,† and Hikaru Mizuno

School of Materials Science, Japan Advanced Institute of Science and Technology, 1-1 Asahidai, Nomi 923-1292

Received April 2, 2010; E-mail: miuray@chem-eng.kyushu-u.ac.jp

We synthesized a novel glycopolymer library with 6-sulfo-GlcNAc and glucuronic acid (GlcA) based on the structure of glycosaminoglycans. The molecular weights of the polymers were controlled via living radical polymerization. The interactions of Aβ(1–40) with glycopolymers were analyzed by inhibition activity of protein aggregation using ThT fluorescence assay, atomic force microscopy observation, and CD spectra. The inhibition activity of Aβ was much affected by the sugar structure and molecular weight of the polymer. The glycopolymers carrying 6-sulfo-GlcNAc showed inhibition activity toward Aβ aggregate, and those with 6-suflo-GlcNAc and GlcA showed the strong inhibition activity. The glycopolymer libraries yielded valuable information about Aβ aggregate with glycosaminoglycans.

Alzheimer's disease (AD) is a serious form of dementia.[1] The defining features of AD are amyloid deposits, neurofibrillary tangles, and selective neuronal loss. The major component of the amyloid deposits is a 39–43 residue peptide, amyloid β (Aβ).[2] Since AD is strongly related to the amyloidosis of Aβ, it is important to clarify the mechanism of formation and inhibition of Aβ aggregates.

It has been reported that the glycosaminoglycans (GAGs) on cell surfaces play important roles in aggregation of Aβ.[3] GAGs interact with Aβ to act as a scaffold for the assembly of fibrils, and the amount of GAGs in the brain tissue from victims of AD is increased. At the same time, oligomers of GAGs have been reported to inhibit Aβ aggregate.[4,5] Consequently, the GAGs–Aβ interaction is a key factor for control of Aβ aggregate, and GAGs-based compounds are potential medicines for AD. It is thus necessary to analyze in detail the mechanism of the GAGs–Aβ interaction. So far, McLaurin et al. reported the effect of GAGs such as heparin, heparan, and chondroitin sulfate on fibril formation.[6] It was confirmed that the GAGs affect the fibril formation of Aβ, but the detailed interaction including the role of saccharides was unclear.

The GAGs are composed of various disaccharide moieties of uronic acid and glucosamine residues, and have highly complex chemical structures due to the diversity of the saccharide component by epimerization, sulfonation, and high molecular weight. The chemical structures of the GAGs are too complex to allow the details of their interaction with Aβ to be elucidated directly, and an ingenious method to analyze the interaction is needed.[7,8] We have reported use of sulfonated glycopolymers as mimic polymers of GAGs, to investigate the GAGs–Aβ interaction.[9] The advantage of glycopolymers is facile preparation, so that the effects of saccharide structure and polymer molecular weight can be easily investigated.

In the present study we synthesized glycopolymer libraries with 6-sulfo-β-GlcNAc and glucuronic acid (GlcA), based on the GAGs structure (Figure 1). The polymers with various molecular weights were also synthesized via reversible addition–fragmentation chain transfer (RAFT) living radical polymerization.[10] The influence of the polymers on Aβ(1–40) aggregate was studied in detail by ThT fluorescence assay, morphology observation, and conformation measurements. Aβ(1–40) was selected in order to analyze the detailed inhibition effect and kinetics.

Experimental

Materials. The following reagents were used as received: amyloid β-protein (Aβ(1–40)) (Bachem AG, Switzerland), 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPD) (Wako Chemical, Osaka, Japan), (thiobenzoyl)thioglycolic acid, hyaluronic acid from rooster comb (Sigma-Aldrich, Louisiana, MO, USA), heparin sodium salt from hog intestine, hexafluoro-2-propanol (HFIP), *p*-nitrophenyl-D-glucose, Pd/C, and thioflavin T (ThT) (TCI, Tokyo, Japan).

Characterization. ^{1}H NMR (300 MHz) spectra were recorded in D_2O at room temperature with a Varian Gemini-

† Present address: Department of Chemical Engineering, Faculty of Engineering, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka 819-0395

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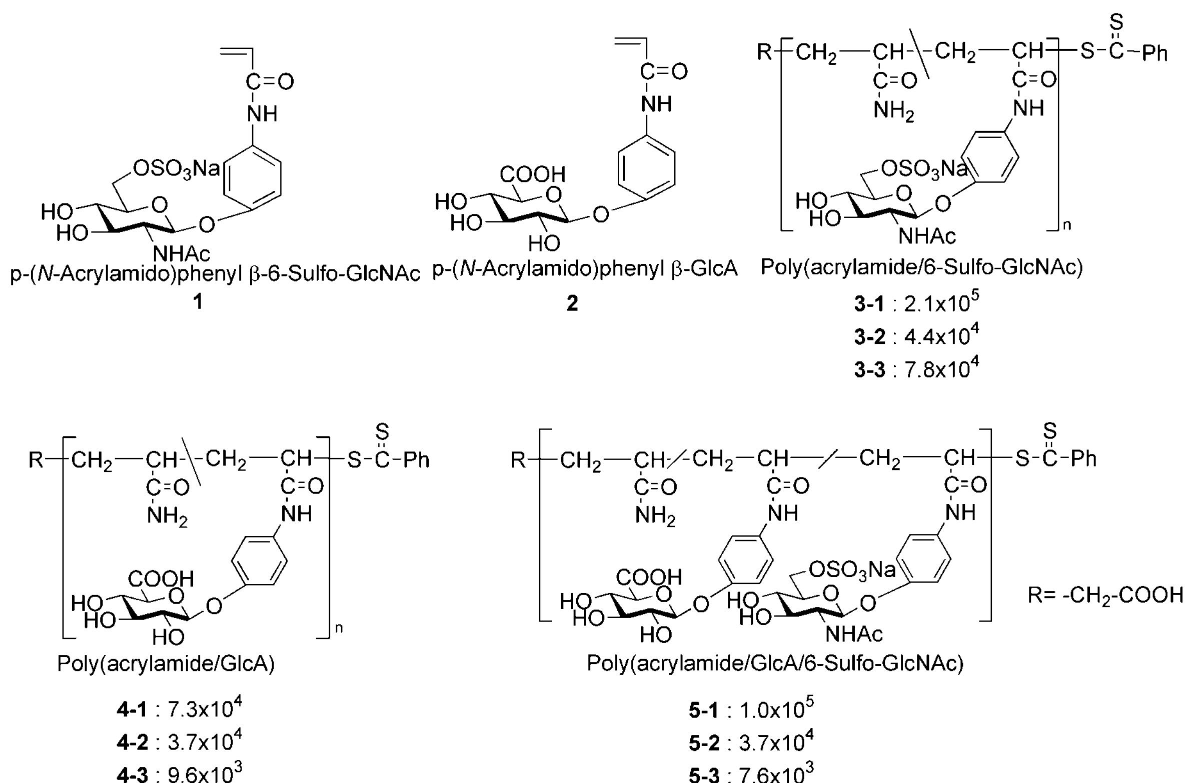


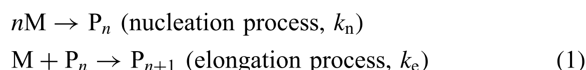
Figure 1. Chemical structures and molecular weights (M_n) of glycopolymers used in this study.

300 spectrometer, and mass spectra were obtained with an ESI-MS LCQ-DECA XP spectrometer (Thermo Scientific, Waltham, MA, USA). Gel permeation chromatography (GPC) was conducted with a JASCO 800 high-performance liquid chromatography instrument on a Shodex SB-804HQ column with PBS as eluent, using a pullulan molecular weight standard. Fluorescence intensity was measured with an LS55 instrument (Perkin-Elmer, Waltham, MA, USA), CD spectra were recorded using a JASCO J-720 spectrometer (JASCO, Tokyo, Japan), and an SPA400 instrument (Seiko Instruments Inc., Tokyo) with 40 N cantilever was used for AFM.

Glycopolymers. *p*-(*N*-Acrylamido)phenyl pyranosides were synthesized via hydrogenation of *p*-nitrophenyl pyranosides, and subsequent treatment with acryloyl chloride.[11,12] Details of the syntheses are given in the Supporting Information. Polymerization was conducted via living radical polymerization using the radical initiator AAPD with (thiobenzoyl)thioglycolic acid as a RAFT reagent.

ThT Fluorescence Assays.[13] Aβ(1–40) was monomerized in HFIP at 2.25 mg mL^{-1} and dried under N_2 at 0 °C, then stored at −80 °C. ThT fluorescence assay was performed for Aβ(1–40) in 50 mM phosphate buffer with 100 mM NaCl at pH 7.5. ThT (20 µM) was added to 100 µL of each Aβ (23 µM) solution in a 96-microwell plate at 37 °C, and the mixtures incubated with shaking at 400 rpm (Incubator FMS, EYELA, Tokyo). The fluorescence intensity was measured with excitation at 450 nm and emission at 485 nm. The time-course of fluorescence was determined via measurements at 1 h intervals.

Kinetic Analyses of ThT Fluorescence Data. ThT fluorescence data were analyzed by the kinetic scheme[14]



$$nM \rightarrow P_n \text{ (nucleation process, } k_n\text{)}$$
$$M + P_n \rightarrow P_{n+1} \text{ (elongation process, } k_e\text{)} \quad (1)$$

where M and P_n denote monomeric and polymeric peptide, respectively. The kinetic constants obtained were k_n and k_e for nucleation and elongation of amyloid fibrils.

ThT data were analyzed in terms of a nucleation dependent polymerization mechanism. This approach considers amyloid fibril formation as an autocatalytic process with nucleation followed by an elongation reaction, with their respective kinetic constants k_n and k_e. Experimental data can be fitted to such a model using eq 2

$$f = \frac{\rho\{\exp[(1+\rho)kt] - 1\}}{\{1 + \rho\exp[(1+\rho)kt]\}} \quad (2)$$

where f is the fraction of the fibrillar form; $k = k_e a$, where a is the initial peptide concentration, and $\rho = k_n/k$. To fit the experimental data to eq 2, fluorescence was converted to fraction of fibril formation, with the condition $f = 0$ at $t = 0$.

AFM Measurements. Aβ(1–40) solution (100 µL, 23 µM) was incubated for 48 h in the presence or absence of glycopolymers in phosphate buffer (50 mM phosphate buffer, 100 mM NaCl, pH 7.5). An aliquot of the sample solution (5 µL) was placed on freshly cleaved mica, rinsed with deionized water (100 µL × 5) and dried. The resultant sample was scanned by D-AFM.

CD Spectra. Aβ(1–40) was monomerized according to the procedure used in the ThT assay. 200 µL of Aβ solution (46 µM) was incubated at 37 °C in a 96 microwell plate with shaking (400 rpm) for 9 days, then used as seed solution.[15]

Table 1. Polymerization of Poly(acrylamide/6-sulfo-β-GlcNAc/GlcA)

No.	M_n a) /g mol^{-1}	M_w a) /g mol^{-1}	M_w/M_n	Conv. /%	Sugar content b) /%	6S-GlcNAc:GlcA	Concentration of RAFT reagent /mol %
3-1	2.1×10^5	2.1×10^5	1.0	67	9.3	1:0	0.10
3-2	4.4×10^4	6.1×10^4	1.4	61	9.6	1:0	0.50
3-3	7.8×10^3	1.0×10^4	1.3	15	12.4	1:0	1.0
4-1	7.3×10^4	1.2×10^5	1.7	81	12.5	0:1	0.10
4-2	3.7×10^4	6.7×10^4	1.8	77	9.2	0:1	0.50
4-3	9.6×10^3	1.2×10^4	1.3	16	13.7	0:1	1.0
5-1	1.0×10^5	1.4×10^5	1.3	81	10.0	1:0.75	0.10
5-2	3.7×10^4	6.8×10^4	1.8	82	10.3	1:0.72	0.50
5-3	7.6×10^3	1.0×10^4	1.4	16	17.4	1:0.89	1.0

a) Relative to pullulan standard. b) By ^{1}H NMR.

1.0 µL of seed solution was added to the Aβ solution (199 µL), which was prepared following the ThT assay solution procedure. The final concentration was 46 µM by UV absorption (280 nm). The solution was incubated for 48 h, and CD spectra were recorded at room temperature using a 2 mm cuvette. Spectra were obtained from 260 to 190 nm at 50 nm min^{-1} scan speed, and 16 spectra were accumulated for each sample.

Results and Discussion

Syntheses of Glycopolymers.[11,12] The syntheses of glycopolymers were conducted via living radical polymerization (Figure 1 and Table 1). The sugar ratio of the polymer was fixed at about 10%, because glycopolymers with modest sugar ratio have been found to show stronger inhibitory effects toward Aβ(1–42) aggregate.[9] The molecular weights of the glycopolymers were of the order 10^3, 10^4, and 10^5 g mol^{-1} with different RAFT reagent ratios. We synthesized the glycopolymers of 6-sulfo-GlcNAc and glucuronic acid (GlcA), and also prepared the terpolymer with 6-sulfo-GlcNAc and GlcA as a glycosaminoglycan mimic polymer.

ThT Fluorescence Assay. Aggregation of Aβ(1–40) was monitored using ThT fluorescence intensity with addition of glycopolymers (Figure 2). Aβ without polymer additives showed a characteristic sigmoidal curve, indicating aggregation with increasing incubation time. The addition of specific glycopolymers changed the time course curve of fluorescence intensity. While addition of the monomer of 6-sulfo-GlcNAc (**1**) was not effective, the addition of glycopolymers with 6-sulfo-GlcNAc (**3-1**, **3-2**, and **3-3**) changed the ThT time course curve. The addition of glycopolymers with 6-sulfo-GlcNAc extended the lag time (Supporting Information), and reduced the final fluorescence intensity. The polymer with lower molecular weight (**3-3**) both reduced the fluorescence intensity and extended the lag time to a greater extent than the polymer with higher molecular weight (**3-1**). The addition of glycopolymer with GlcA (**4-1**, **4-2**, and **4-3**) did not result in significant changes in the ThT intensity vs. time curve; the lag time was almost the same as that without additives. By contrast, the glyco-terpolymers with 6-sulfo-GlcNAc and GlcA (**5-1**, **5-2**, and **5-3**) induced drastic changes in the ThT curve; the fluorescence intensity was decreased by 70% and the lag time increased. In particular, the terpolymer of **5-3** was the best inhibitor of all the glycopolymers.

Kinetic Analyses. ThT fluorescence traces were kinetically fitted in terms of amyloid nucleation (k_n) and elongation (k_e) rate constants (Table 2). The glycopolymers with 6-sulfo-GlcNAc (**3-1**, **3-2**, and **3-3**) showed smaller k_n and larger k_e, by comparison with the ThT curve without additives. The glycopolymer with smaller molecular weight (**3-3**) showed smaller k_n, and larger k_e, hence inhibition of nucleation and acceleration of amyloid fibril elongation. In the case of glycopolymers with GlcA (**4-1**, **4-2**, and **4-3**), somewhat larger k_n and smaller k_e were found. The terpolymers with both 6-sulfo-GlcNAc and GlcA (**5-1**, **5-2**, and **5-3**) showed the smallest k_n and modest k_e values, signifying inhibition of amyloid nucleation and fibril formation. These results indicated that the saccharide structure of GAGs was strongly correlated with amyloidosis. The kinetic analyses suggested that the terpolymer with 6-sulfo-GlcNAc and GlcA was effective for Aβ aggregate. The molecular weight of the polymers also had an effect on the aggregation behavior of Aβ; low molecular weight glycopolymers exhibited smaller k_n.

Those results indicated that the binding of sulfonated GlcNAc to Aβ inhibited nucleation but contributed to fibril formation. The binding of glycopolymers to Aβ was considered to be occurred via an electrostatic interaction between the sulfonated groups of the 6-sulfo-GlcNAc and the basic residue in the HHQK domain.[16,17] The glycopolymers without sulfonated group did not inhibit the aggregation of Aβ in this experiment and our previous research.[9] The binding of Aβ to sulfonated glycopolymer inhibited the binding to other Aβ, which inhibited nucleation and amyloidosis. At the same time, the sulfonated GlcNAc contributed to fibril formation, which was consistent with the previous report that the addition of GAGs induces the fibril formation of Aβ due to the scaffold effect for fibril formation.[6] Since the sugar contents of the glycopolymers were modest, the glycopolymers did not act as a scaffold for the fibrils assembly and did not induce the extensive aggregation.

GlcA residue showed weak interaction with Aβ, and a little acceleration of nucleation. The acidity of GlcA was too weak to interact with basic amino acid residues, and so the glycopolymer with GlcA was not effective inhibitor of Aβ aggregate. Rather, the glycopolymer with GlcA might provide a nucleation field for Aβ. On the other hand, the terpolymers (**5-1**, **5-2**, and **5-3**) were the most suitable for the inhibitory effect. It is

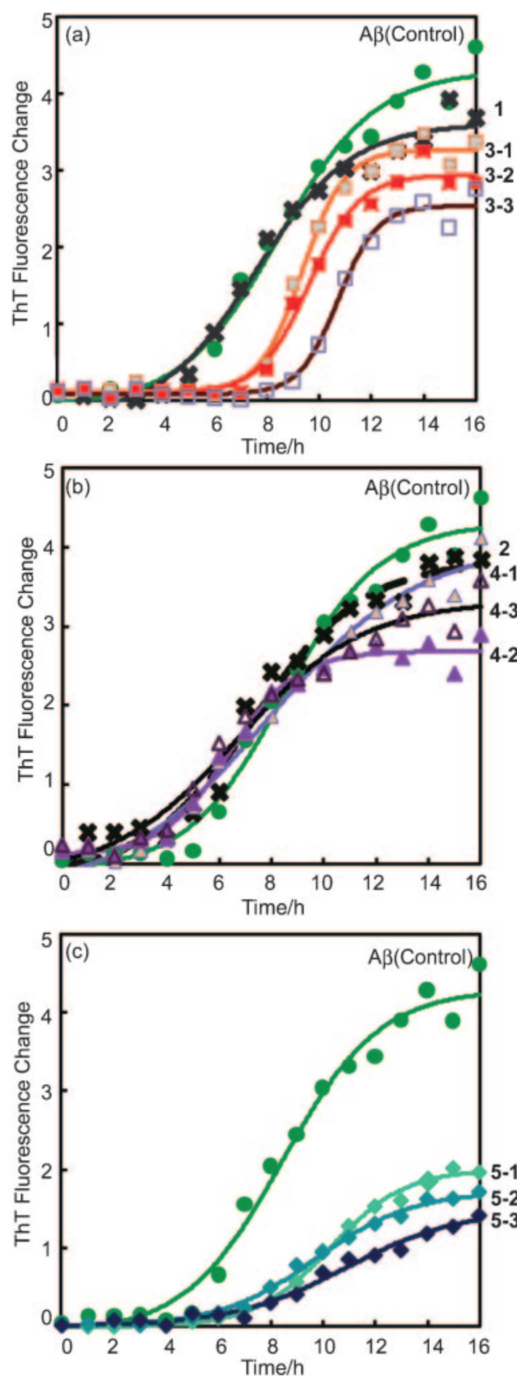


Figure 2. Time course of fibril formation at pH 7.5 and 37 °C with 400 rpm shaking monitored by ThT fluorescence. The concentrations of Aβ and sugar were 23 and 200 µM, respectively. The sugar additives were (a) poly(acrylamide/6-sulfo-GlcNAc), (b) poly(acrylamide/GlcA), and (c) poly(acrylamide/GlcA/6-sulfo-GlcNAc).

noteworthy that the polymers having the most suitable mimic structure with uronic acid and sulfonated GlcNAc showed the best inhibitory effects.

The glycosaminoglycans are composed of uronic acid and sulfonated glucosamine residues.[18] Consequently, the proteins might have affinity not only to the sulfonated glucosamine but also uronic acid portion. The binding to GlcA is expected to be

Table 2. Nucleation and Elongation Rate Constants Calculated by Fitting ThT Data

Sample	k_n/s^{-1}	k_e/L mol^{-1} s^{-1}
Aβ (control)	1.3×10^{-6}	3.4
1	1.4×10^{-6}	3.7
3-1	7.3×10^{-9}	6.6
3-2	2.5×10^{-8}	5.9
3-3	5.1×10^{-10}	7.9
2	3.4×10^{-6}	3.0
4-1	4.2×10^{-6}	2.5
4-2	2.5×10^{-6}	4.2
4-3	6.7×10^{-6}	2.5
5-1	1.3×10^{-6}	4.3
5-2	6.7×10^{-7}	3.4
5-3	6.8×10^{-7}	3.1
Heparin	3.1×10^{-6}	3.7
Hyaluronic acid	1.5×10^{-6}	3.4

weak compared to 6-sulfo-GlcNAc based on the ThT results of **3-3** and **4-3**. But the cooperative binding to 6-sulfo-GlcNAc and GlcA is advantageous to the Aβ binding.[19] Thus, the glycopolymers with 6-sulfo-GlcNAc and GlcA (**5-1**, **5-2**, and **5-3**) showed a better inhibition effect on Aβ aggregate than those with 6-sulfo-GlcNAc (**3-1**, **3-2**, and **3-3**).

The molecular weight dependence was significant, and the low molecular weight polymers showed the stronger inhibition effects. It has also been reported that the low molecular weight heparin inhibited Aβ aggregate.[4,5] Molecular weight changes affect the physical properties of the polymers such as mobility,[20,21] and the low molecular weight polymers had better mobility. It has been reported that glycopolymer with better mobility showed better binding properties to the target protein,[22] and so the greater mobility could contribute to the better binding. In addition, we fixed the concentration of sugars in the experiments, and the number of the molecules was different. That is, the low molecular weight of polymers had a larger number of molecules than did the high molecular weight polymers, though the sugar concentration was the same. The difference in number of molecules could contribute to binding by the entropy effect, which gives better binding of low molecular weight polymers.[23] Detailed analyses of the molecular weight effect are still under investigation, but the glycopolymers with smaller molecular weights definitely had better affinities to Aβ, resulting in inhibition of nucleation. Since the monomer did not show the inhibitory effect, the multivalent sugar compounds with low molecular weight were the best design for inhibition of Aβ aggregate.

AFM Measurements. The morphologies of Aβ with various additives were investigated by AFM; representative data are shown in Figure 3. The Aβ aggregates without additives formed amyloid fibrils with length 1–3.5 µm, width 160–230 nm, and height 15–45 nm (Figure 3a). The glycopolymers with 6-sulfo-GlcNAc (**3-1**, **-2**, **-3** and **5-1**, **-2**, **-3**) changed the morphology to smaller amyloids. In addition, the morphology change was clearly dependant on the molecular weight of the glycopolymer additives: the additives with smaller molecular weight showed better inhibitory effects on Aβ aggregate. For example, the Aβ aggregates with **3-1** were 0.48–2.8 µm long, 80–180 nm wide, and 6–35 nm high, and

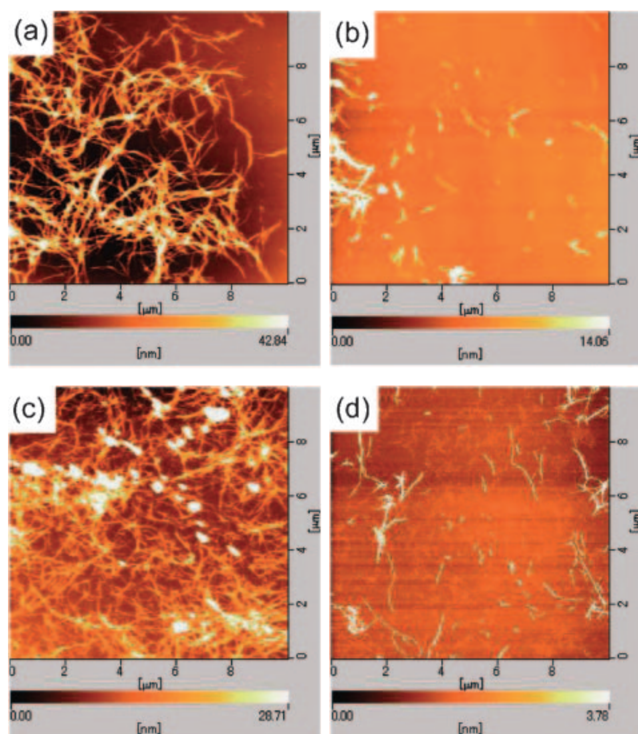


Figure 3. AFM images of the aggregates of Aβ(1–40). Amyloid fibrils were obtained by incubation of Aβ (23 µM) at pH 7.5 and 37 °C with 400 rpm shaking for 48 h. The images were (a) without glycopolymer, (b) in the presence of **3-3** (200 µM), (c) in the presence of **4-3** (200 µM), and (d) in the presence of **5-3** (200 µM).

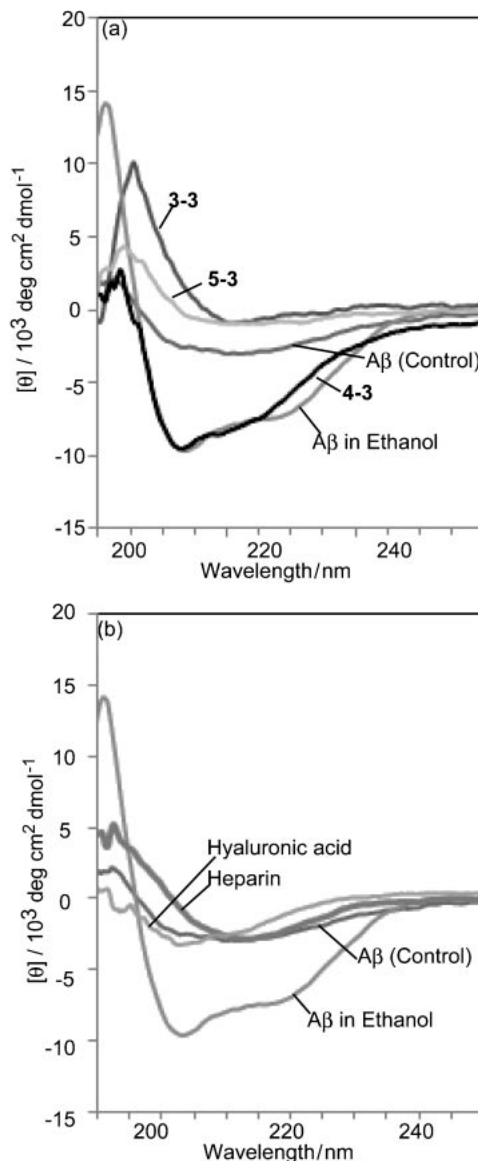


Figure 4. CD spectra of Aβ(1–40). The Aβ solution was prepared by the incubation of Aβ (46 µM) at pH 7.5 and 37 °C with 400 rpm shaking for 48 h. The CD spectra were (a) in the presence of glycopolymers of **3-3**, **4-3**, and **5-3** at 400 µM, and (b) in the presence of polysaccharide of hyaluronic acid and heparin at 1.0 mg mL^{-1}.

those with **3-3** had length 0.19–2.0 µm, width 110–180 nm, and height 1–8 nm (Figure 3b). Glycopolymers with both 6-sulfo-GlcNAc and GlcA (**5-1**, **5-2**, and **5-3**) changed the morphologies to the large extent. Aβ with **5-3** strikingly reduced the size of the amyloids to 0.10–1.7 µm length, 80–120 nm width, and 1.5–4.0 nm height (Figure 3d). On the other hand, the glycopolymers without 6-sulfo-GlcNAc (with only GlcA, **4-1**, **4-2**, and **4-3**) did not show the changes in morphology that were visible by AFM observation (Figure 3c). Those observations were consistent with the results of the ThT fluorescence assay.

CD Spectra Measurement. The conformation of Aβ was determined with glycopolymers (Figure 4).[24] Aβ without additives showed a negative Cotton effect around 220 nm, suggesting a β-sheet structure. Addition of glycopolymers induced specific conformations in Aβ (Figure 4a). Glycopolymers with 6-sulfo-GlcNAc (**3-1**, **3-2**, and **3-3**) gave a β-sheet structure for which the intensity of the Cotton effect was smaller than for the control sample. In the case of ter-glycopolymers (**5-1**, **5-2**, and **5-3**), the spectra showed broad negative Cotton effects with weaker intensity.

Interestingly, the glycopolymers with GlcA (**4-1**, **4-2**, and **4-3**) induced a different conformation: the CD spectra showed a negative Cotton effect at 208 and 218 nm, suggesting a helical structure. We also measured the CD spectra of Aβ in ethanol solution to induce the helical structure due to the hydrophobicity,[24] and the intensity of the Cotton effect around 208 nm was almost the same as that with **4-3**. The clear difference in conformation showed that the interactions of Aβ with GAGs affected amyloidosis and induced specific conformations.

We examined the conformation of Aβ with addition of natural GAGs of heparin (possessing 6-sulfo-GlcNAc) and hyaluronic acid (possessing GlcA). The addition of real GAGs also changed the conformation of Aβ (Figure 4b). Heparin induced a negative Cotton effect at 218 nm, hence gave rise to a β-sheet structure, and the addition of hyaluronic acid induced a broad negative Cotton effect at 208 nm, suggesting a different conformation from β-sheet. The CD spectra with GAGs reflected those with model glycopolymers. Comparing the sulfonated GAGs (such as heparin and heparan), attention was not paid to the role of uronic acid and hyaluronic acid due to the weak interaction. If the polyvalent uronic acid or hyaluronic

acid can operate as a molecular chaperon in amyloidosis,[25] the molecules could be useful bioactive compounds.

The correlation between secondary structure by CD and aggregation properties were contrary to the previous reports. It has been reported that the amyloid fibrils are composed of β-sheet rich proteins. However, in our results with glycopolymers, the Aβ with β-sheet structure in the presence of **3-3** and **5-3** showed less aggregation properties with ThT and AFM. The Aβ with helical structure in the presence of **4-3** showed strong aggregation properties by ThT.

It has been suggested that the conformation of Aβ was affected by the HHQK region.[16,17,26] It is possible that the binding of 6-sulfo-GlcNAc to the HHQK domain changes the conformation of Aβ into β-sheet. However, at the same time the strong binding of glycopolymer to Aβ inhibited the aggregation between each Aβ peptide. In this experiment, the strong binding of Aβ was more important than secondary structure in inhibition of Aβ aggregate. Hence, in the presence of glycopolymer, Aβ with β-sheet like structure showed weaker aggregative properties.

Conclusion

We investigated the syntheses and biological properties of GAGs mimic polymers for Aβ. The addition of GAGs mimic polymers produced specific properties based on the pendant saccharide structures. An appropriate glycopolymer with saccharide structure and suitable molecular weight can control the amyloidosis and conformation of Aβ. These approaches provide information on the function of GAGs in relation to Alzheimer's disease. The detailed functionalities of GAGs in AD are still unclear, but our experiments with glycopolymers could reveal partial functionalities.

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Supporting Information

The synthetic procedure of polymers, AFM, CD data, and the schematic description of ThT assay. These materials are available free of charge on the web at http://www.csj.jp/journals/bcsj/.

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