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NOTE

Glycosaminoglycan model polymers with Poly(γ-glutamate) backbone to inhibit aggregation of β-Amyloid peptide

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INTRODUCTION

Alzheimer's disease is a serious dementia frequently accompanied by the emergence of senile plaques in the cerebral mesothelium. Senile plaques involve pathogenic aggregates of β-amyloid peptide (Aβ) with a β-sheet structure.[1] Aβ aggregates deposit on the cell via interactions with extracellular matrices such as glycosaminoglycans (GAGs). GAGs have received much attention with respect to Alzheimer's disease pathogenesis, and it has been found that the amount of bound Aβ is related to the GAG structure.[2–4] GAGs have a negative net charge based on the sulfate group and carboxyl group on the saccharide. The biological activities of GAGs vary according to the saccharide structure in the GAG; such activities include interactions with Aβ, and these cause Aβ intricate changes. Although the addition of GAGs inhibits the aggregation of Aβ in some cases, GAGs sometimes induce Aβ aggregation, with conformational changes.[5–8] It is important to clarify the complex biological activity of GAGs in order to elucidate the pathogenic mechanism with respect to Aβ and to develop medicinal compounds based on GAGs. It has been reported that heparin, which is a GAG, inhibits Aβ aggregation as a result of the electrostatic interactions between the anionic sulfate group of heparin and the cationic domain of Aβ13–16(HHQK).[9] The abundant sulfate groups of heparin and other GAGs are the key to their interactions with Aβ, but the interactions between GAGs and Aβ are difficult to analyze because GAGs have complicated saccharide structures and high molecular weights. To overcome the difficulties of GAG analysis and enable facile development of therapeutic materials, we previously investigated GAG-mimicking polymers with multivalent sulfated saccharides, and reported that glycopolymers with sulfonated saccharides interacted with Aβ and controlled its aggregation.[10–12] The usage of GAGs mimic with well-ordered structures and molecular weights helped to analyze the molecular interaction in detail, and we have clarified that Aβ binds to sulfonated saccharides by electrostatic interactions. We also prepared GAG-mimicking polymers with multivalent uronic acids (a sugar with a carboxylic acid group) and found that the carboxylic acid on uronic acid also delays elongation in Aβ aggregation.[11] A glyco-copolymer of uronic acid and a sulfated saccharide, which showed strong inhibition of Aβ activity, has been synthesized. These results indicated that GAG-mimicking polymers with two anionic groups, namely carboxylic acid and sulfate groups, are good inhibitors for Aβ. These glycopolymers had an acrylamide backbone and were synthesized via radical polymerization. Synthesis of glycopolymers by radical polymerization is a facile method of preparing versatile GAG-mimicking polymers.[13] However, it was suspected that the biocompatibility of the synthetic polyacrylamide backbone was insufficient.[14]

In this research, we focused on the synthesis and properties of novel GAG-mimicking polymers with a poly(γ-glutamate) (PGA) backbone. PGA is known to have good biocompatibility. As PGA is a natural product, not only PGA itself but also PGA hydrolysates are biocompatible.[15,16] In addition, PGA is highly water soluble, which is appropriate for polymeric drugs. The important point of this study is the use of PGA as a glycopolymer backbone because of its biocompatibility. We modified PGA with *p*-nitrophenyl-6-sulfo-*N*-acetyl-D-glucosamine (6S-GlcNAc: **0-A**) and taurine to give GAG-mimicking polymers with both carboxyl and sulfate groups, and studied the inhibitory effects of the GAG-mimicking PGA polymers.

EXPERIMENTAL PROCEDURE

Synthesis of PGA derivatives

6S-GlcNAc **0-A** was synthesized as in previous research.[12] GAG-mimicking polymers were synthesized by introducing the sulfate compounds 6S-GlcNAc (**1** series) and taurine (**2**) into PGA (M_w 1.07×10^5 (based on pullulan standard), $M_w/M_n = 1.67$, Wako Pure Chemical Industries, Osaka, Japan) via condensation reactions (Figure 1). The introduction rates of PGA carboxyl

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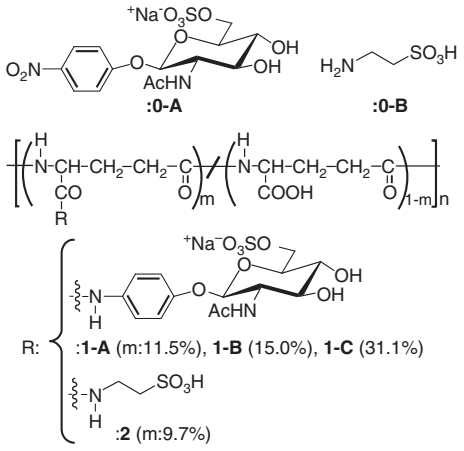

Figure 1 The molecular structures of 6S-GlcNAc (**0-A**), taurine (**0-B**) and GAGs mimic polymers with PGA; 11.5% substitution with 6S-GlcNAc (**1-A**), 15.0% substitution with 6S-GlcNAc (**1-B**), 31.1% substitution with 6S-GlcNAc (**1-C**) and 9.7% substitution with taurine (**2**).

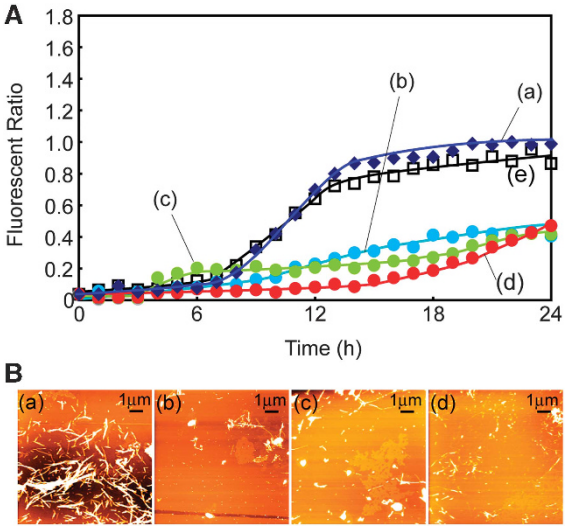

Figure 2 (**A**) Fluorescent ratio curves of Aβ (1–40) without additives (closed diamond (a)) in th-T assay, set the fluorescence at 24 h as the standard value. Other curves suggested that the fluorescent ratios of Aβ (1–40) with 500 μM (as saccharide concentration) saccharide derivatives of **1-A** (closed blue-circle (b)), **1-B** (closed green-circle (c)), **1-C** (closed red circle (d)) and **0-A** (open box (e)). (**B**) Aggregate morphologies of Aβ (1–40) (a), with 500 μM (as saccharide concentration) **1-A** (b), **1-B** (c) and **1-C** (d) after 48 h incubation.

groups were calculated by ^{1}H-NMR analysis to be 11.5% for **1-A**, 15.0% for **1-B**, 31.1% for **1-C** and 9.7% for **2**. The molecular weights of the complexes after modification were estimated to be $4.00 \times 10^5/6.23 \times 10^5$ (M_w/M_n) for **1-A,** $3.12 \times 10^5/6.34 \times 10^5$ (M_w/M_n) for **1-B,** $3.39 \times 10^5/6.58 \times 10^5$ (M_w/M_n) for **1-C** and $5.29 \times 10^5/7.48 \times 10^5$ (M_w/M_n) for **2,** using GPC system with a pullulan standard in PBS$^-$ buffer at pH 7.4.

Evaluation of Polymer Function on Aβ aggregation

Aβ (1–40) was monomerized in hexafluoro-2-propanol. The aggregation of Aβ (1–40) was monitored by the fluorescence of thioflavin-T (th-T). Th-T places between β-sheet aggregations of Aβ (1–40). Th-T fluorescence assay was performed for 23 μM Aβ (1–40) in 50 mM phosphate buffer with 100 mM NaCl at pH 7.4 in the presence and in the absence of polymer additives. The aggregation was monitored by the periodical measurement of fluorescence (excitation wavelength 450 nm and emission wavelength 485 nm). The morphology of Aβ (1–40) was monitored by tapping-mode atomic force microscopy (AFM; SPI3800N instrument; Seiko Instruments Inc., Chiba, Japan) equipped with an SPA400 as a probe station, and a DF40P cantilever using each sample after 48 h incubation. Each sample was dropped on freshly cleaved mica and was dried by the spontaneous evaporation within a few minutes. The sample loaded mica was monitored after gently rinsed by distilled water and dried by the spontaneous evaporation.

RESULTS AND DISCUSSION

Analyses with GAG-mimicking glycopolymers

The inhibitory activities of the GAG-mimicking glycopolymers for Aβ (1–40) aggregation were evaluated from the fluorescent ratio changes with the thioflavin-T (th-T) assay. The time-courses of th-T assays using **0-A** and the **1** series (500 μM) are illustrated in Figure 2A. The time-course of the fluorescence changes without additives showed a gradual increase for 24 h of incubation, indicating aggregation of Aβ, and the lag time before a steep increase in fluorescence was around 6 h. The fluorescence time-course with monomeric 6S-GlcNAc (**0-A**) was almost the same as that without additives, showing no inhibition of Aβ aggregation.

The fluorescence time-course for 6S-GlcNAc-modified PGA (**1** series) showed drastic changes; the lag times became longer and the fluorescence decreased by <48%. The maximum fluorescence of each sample with a GAG-mimicking glycopolymer was almost the same at 24 h, but the increase in the curve for the low level of glycopolymer introduction was more gradual than those for higher levels of glycopolymer introduction, although there were some differences. The addition of PGA without saccharide modification did not result in inhibitory activity. These results indicate that glycopolymers of PGA with 6S-GlcNAc inhibited Aβ aggregation by multivalent sulfated saccharide–Aβ interactions.

The morphologies of Aβ with/without additives after 48 h of incubation were observed using AFM (Figure 2B). Aβ without additives formed fibrils, of estimated widths, heights and lengths of 9–30 nm, 93–180 nm and 0.97–2.50 μm, respectively. The Aβ morphology with glycopolymer **1-A** consisted of amorphous aggregates smaller than those of the control (Figure 2Bb), with the widths, heights and lengths estimated to be 2–13 nm, 53–66 nm, and 0.62–1.66 μm. Aβ with glycopolymers **1-B** and **1-C** also formed amorphous aggregates, and the widths, heights and lengths, respectively, were 4–8 nm, 41–290 nm and 0.30–0.56 μm, and 4–10 nm, 131–364 nm and 0.08–0.75 μm. In contrast, the addition of monomeric 6S-GlcNAc (**0-A**) hardly affected the morphology, and the shape was the same as that without additives.

Circular dichroism spectroscopy of Aβ (1–40) with/without GAG-mimicking glycopolymers was also performed (see Supplementary Information). The band intensity around 218 nm of Aβ with a GAG-mimicking polymer was smaller than that without additives, and suggested a decrease the β-sheet structure, based on interactions between Aβ (1–40) and the GAG-mimicking glycopolymer. Overall, glycopolymers with a PGA backbone with 6S-GlcNAc showed excellent inhibition of Aβ aggregation, similar to inhibition by GAGs. The GAG-mimicking polymers **1-A** and **1-B** also showed significant dose-dependency on Aβ aggregation, determined by th-T assays (Figure 3). The maximum fluorescence intensity decreased with increasing glycopolymer concentration from 100–500 μM; the intensities for **1-A** and **1-B** were 0.66–0.40 and 0.80–0.42, respectively.

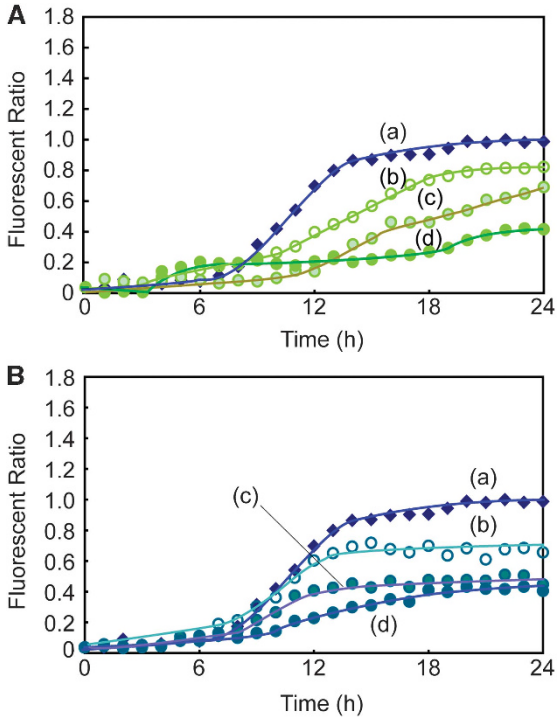

Figure 3 (**A**) Fluorescent ratio curves of Aβ (1–40) without additives (closed diamond (a)) in th-T assay, set the fluorescence at 24 h as the standard value. Other curves suggested that the fluorescent ratios of Aβ (1–40) with 100 μM (open circle (b)), 250 μM (open circle and filled with light blue in the center (c)), 500 μM of **1-A** (closed blue-circle (d)). Each concentration was calculated as saccharide concentration. (**B**) Fluorescent ratio curves of Aβ (1–40) without additives (closed diamond (a)) in th-T assay, set the fluorescence at 24 h as the standard value. Other curves suggested that the fluorescent ratios of Aβ (1–40) with 100 μM (open circle (b)), 250 μM (open circle (b) and filled with light blue in the center (c)), 500 μM of **1-B** (closed blue-circle (d)). Each concentration was calculated as saccharide concentration.

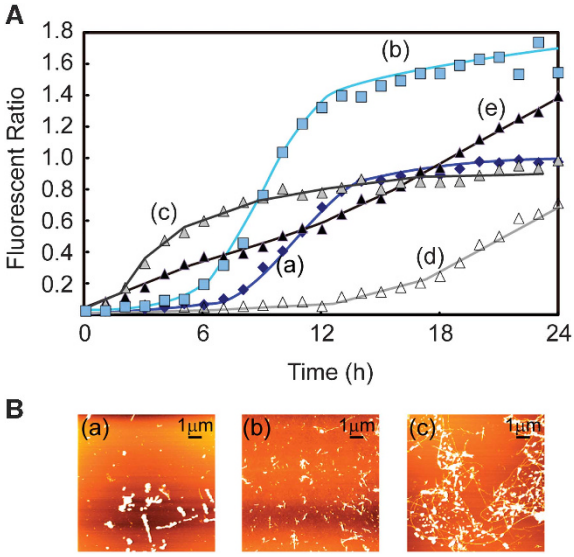

Figure 4 (**A**) Fluorescent ratio curves of Aβ (1–40) without additives (closed diamond (a)) in th-T assay, set the fluorescence at 24 h as the standard value. Other curves suggested that the fluorescent ratios of Aβ (1–40) with 500 μM **0-B** (closed box (b)), and with taurine polymer **2** of 100 μM (open triangle (c)), 250 μM (closed gray-triangle (d)), 500 μM (closed black-triangle (e)). Each concentration was calculated as taurine concentration. (**B**) Aggregate morphologies of Aβ (1–40) with 100 μM (as taurine concentration) (a), 250 μM (b) and 500 μM (c) of **2** after 48 h incubation.

Analyses with GAG-mimicking taurine polymer

The results in Figures 2 and 3 indicate the importance of the sulfated saccharide portion in interactions with Aβ. As the key to the inhibitory effect is considered to be the electrostatic interactions between Aβ and sulfated saccharides, it was expected that the basic requirement of the GAG mimic was a multivalent sulfate group. We therefore synthesized a new GAG mimic using taurine (**0-B**). Taurine (2-aminoethanesulfonic acid) is a small compound with a sulfate group, and is known to be biocompatible.[17,18] Taurine has been reported to have a little inhibitory activity for Aβ peptides.[19] A GAG-mimicking polymer modified with taurine was synthesized by the same method as that used for the GAG-mimicking glycopolymers.

The time-courses of th-T fluorescence changes of Aβ with **0-B** and **2** were different from both those for Aβ without additives and those with 6S-GlcNAc; the taurine and taurine-modified GAG mimics induced rather complex behavior compared with that without additives (Figure 4A). The maximum fluorescence with **0-B** reached 1.75 at 24 h, suggesting that taurine induced Aβ aggregation (Figure 4Ab). The fluorescence change of Aβ with 250 μM **2** also suggested an inhibitory effect on aggregation (maximum fluorescence decreased 0.73 (Figure 4Ad)). However, the fluorescences with 100 μM and 500 μM **2** showed that there was no inhibitory effect (Figures 4Ac,d), but rather that aggregation was accelerated. The former showed the twice shortened lag time before the elongation process, although the maximum fluorescence was as almost same as that without additives (0.98). The latter showed the larger maximum fluorescence than that without additives (1.41), although the same lag time as that without additives. The results indicated that the addition of **2** sometimes inhibited and sometimes induced Aβ aggregation by its concentration. These nonlinearly aggregations were often observed in the amyloid inhibition assay.[10] The morphology of Aβ with **2**, observed using AFM, is illustrated in Figure 4B. The largest amount of aggregation occurred with addition of 500 μM **2** (Figure 4Bc, widths: 4–20 nm, heights: 79–160 nm and lengths: 0.18–1.31 μm); this is consistent with the results of the th-T assay. In contrast, Aβ with lower concentrations (100 and 250 μM) of **2** showed little aggregation (Figure 4Ba, widths: 3–81 nm, heights: 92–330 nm and lengths: 0.13–1.84 μm; Figure 4Bb, widths: 2–12 nm, heights: 68–173 nm, lengths: 0.32–0.67 μm). AFM observations also suggested that the GAG-mimicking taurine polymer **2** inhibited Aβ aggregation at low concentrations, but became a promoter of Aβ aggregation at higher concentrations, in contrast to the results using GAG-mimicking glycopolymers.

The sulfated polysaccharides inhibit and in contrast, also promote Aβ aggregation. Acceleration of Aβ aggregation by the sulfated polymers was explained as follows. The tangled Aβ cluster formed by the sulfate acted as a seed for the nucleation process, and strongly induced aggregation, involving other Aβ units, in the subsequent elongation process. Acceleration of Aβ aggregation at higher concentrations of the taurine polymer suggested that the Aβ seed interacted with another Aβ, in contrast to the situation with glycopolymers. The results implied different mechanisms of Aβ aggregate formation, depending on the type of GAG-mimicking polymer. GAG-mimicking glycopolymers were better aggregation inhibitors than the GAG-mimicking taurine polymer.

CONCLUSION

In conclusion, we synthesized biocompatible GAG-mimicking polymers containing both sulfate and carboxyl groups by combining PGA with sulfonates. The GAG-mimicking glycopolymers were effective as Aβ aggregation inhibitors, especially at higher concentrations, but the GAG-mimicking taurine polymer often increased Aβ aggregation.

Electronic supporting information available

The detailed syntheses of GAGs-mimicking glyocopolymer and taurine polymer, Circular dichroism spectrum with/without GAGs-mimicking glycopolymer.

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Supplementary Information accompanies the paper on Polymer Journal website (http://www.nature.com/pj)