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5

Interaction and Aggregation of Amyloid β Peptide with Multivalent Sulfonated Sugar

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1. Introduction

Proteins have native functional states but can also form amyloid fibrils (Fowler et al .,2007, Maji et al., 2009, Dobson et al., 2003). In the amyloid state, proteins are denaturated and aggregated with β-sheet-rich structures. Accumulation of amyloids can result in protein amyloidosis, which has attracted much attention. Amyloidosis is associated with many serious neurodegenerative diseases, such as Alzheimer's disease (Sipe et al., 1992), dialysis-related amyloidosis (Rochet et al., 2000), prion disease (Prusiner, 1998), and type II diabetes (Ahmad et al., 2004). Consequently, it is important to clarify the mechanism of amyloidosis, so that it can be inhibited or controlled. Amyloid structures have been analyzed by various methods including nuclear magnetic resonance (NMR) spectroscopy, electron microscopy, X-ray spectroscopy and Fourier transform infrared (FTIR) spectroscopy. Theoretical calculations of amyloidosis have also been conducted (Miller et al., 2010).

Protein amyloidosis is affected by environmental conditions such as the temperature (Kusumoto et al., 1998), pH (Petkova et al., 2004), and ionic strength (Zidar et al., 2011), and by nucleation (Kanji et al., 2008). Because amyloidosis occurs at the cell surface, the interaction between the protein and cell interface is also important (Xu et al., 2005). Therefore, the amyloidosis of proteins can be controlled by manipulating the environmental conditions. However, it has been reported that artificial additives, such as metal ions (Chanki et al., 2007), peptides (Suzuki et al., 2010), sugars (Anubhav et al., 2004) and nanoparticles (Saraiva et al., 2010), can alter the environmental conditions and affect amyloidosis.

Because amyloidosis occurs *in vivo*, the molecules on the cell surfaces are of interest as additives to control amyloidosis. It has been reported that protein amyloidosis with Amyloid beta (Aβ) (Alzheimer's disease) (McLaurin et al., 1996), β2-microglobulin (dialysis-related amyloidosis) (Bourgault et al., 2011), and prion protein (Pan et al., 2002) formation is affected by interactions with glycosaminoglycans (GAGs) on the cell surfaces. This affects all these proteins, even though they have different amino acid sequences and native protein structures. GAGs are long unbranched polysaccharides of repeating disaccharide subunits of hexosamines (glucosamine and galactosamine) and uronic acid (glucuronic acid and iduronic acid) (Rudd et al., 2010). Well-known GAGs are heparin, chondroitin sulfate, keratin sulfate, dermatan sulfate and hyaluronic acid. Most GAGs are highly sulfonated. The interaction of proteins with GAGs is important in amyloidosis, but it is difficult to analyze the detail of this interaction because GAGs have complex structures and high molecular weights.

Synthetic model molecules are useful to clarify the biological function of GAGs. Suda et al. used a synthetic pentasaccharide that bound antithrombin III to study the function of heparin, and produced a biochip with the synthetic GAG (Suda et al., 1993, 2006). Although a number of studies have been reported the compositions of oligosaccharides in GAGs (Jose et al., 2006) and syntheses for GAGs (Jeroen et al., 2005), these investigations did not consider the effect of the molecular weight of GAGs even though they have high molecular weights (5,000–30,000). It is difficult to account for the physical properties and multivalent effects of saccharides within the oligosaccharide following polymerization.

We previously investigated the biological functions using multivalent glycoclusters, and applied these results to production of materials (Miura et al., 2007). Multivalency is important in the biological function of saccharides because it increases the affinity between the saccharides and proteins when the saccharide interaction is weak (Mammen et al., 1998). Glycoclusters include glycopolymers, glycodendrimers (Aoi et al., 1995), and glyco-thin layers (Lang et al., 2008), which all increase the protein–saccharide interactions. Among these types of glycoclusters, glycopolymers with pendant saccharides showed the strongest amplification effect. In this work, we investigated the interaction between GAG models and Aβ to examine the function of GAGs in amyloidosis. The saccharide used was 6-sulfo-*N*-acetyl-glucosamine (6S-GlcNAc), which is frequently present in heparin (Uchimura et al., 1998, Sasaki et al. 2003). An artificial glycocluster of glycopolymer and a glyco-thin layer was prepared, and its interaction with Aβ was investigated.

2. Inhibition of aggregation of amyloid β with sulfonated glycopolymers

In this section, synthetic glycopolymers were produced to mimic GAGs and used to investigate control of aggregation of Aβ. Although the saccharide–protein interaction is generally weak, the glycopolymer increases this interaction through its multivalency. The multivalency can be easily altered by adjusting the initial feed ratio in the polymerization. It is difficult to complete the total synthesis of GAGs. In the present study, GAGs were mimicked using a glycopolymer (Figure 1) that was an acrylamide derivative of the saccharide 6S-GlcNAc.

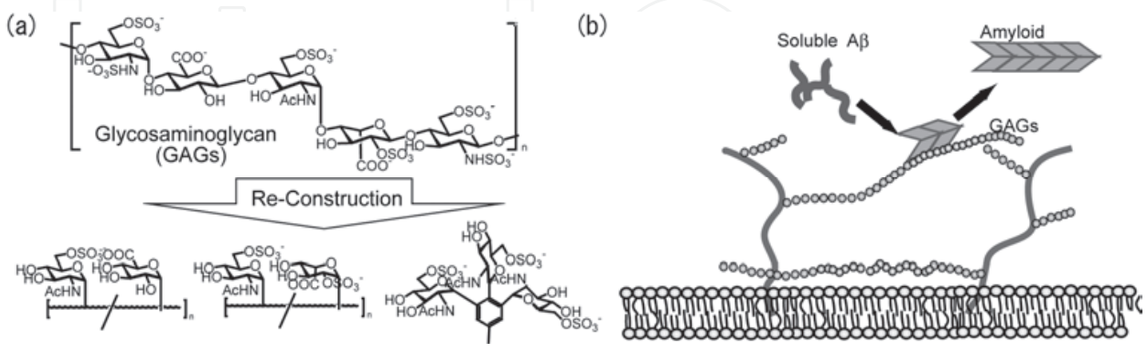


Fig. 1. The GAGs in amyloidosis and this chapter's study. (a)The design of GAGs mimic compounds. (b) The role of GAGs in amyloidosis.

The monomer was synthesized from *N*-acetyl-glucosamine (GlcNAc) by modification with *p*-nitrophenyl-GlcNAc. The obtained acrylamide derivative was polymerized using a radical

initiator. The saccharide contents were varied in the copolymerization of glycopolymer and acrylamide. The sugar contents of the polymers were 10–100 %, and the molecular weights of the polymers were in the order of 10^5. The reference polymers were the glycopolymer of GlcNAc without sulfonated GlcNAc, polyacrylamide, and monomeric 6S-GlcNAc (Figure 2). The interactions of Aβ with the glycopolymers were evaluated by a Thioflavin T (ThT) fluorescence assay, observation of the morphology by atomic force microscopy (AFM), circular dichroism (CD) spectroscopy, and *in vitro* neutralization of an MTT assay. The protein used in this section was Aβ(1-42).

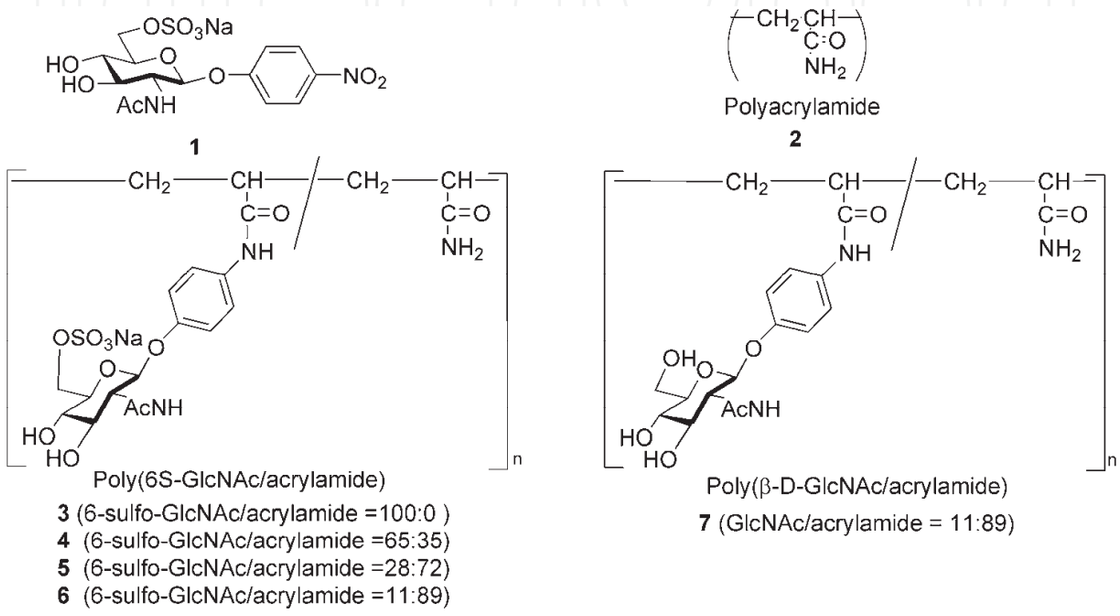


Fig. 2. Chemical structure of GAGs mimic polymers used in section 2

2.1 ThT fluorescence assay of Aβ aggregation in the presence of glycopolymers

The aggregation of Aβ was monitored with ThT in the presence of different glycopolymers (Figure 3). ThT binds to β-sheet aggregates. The ThT fluorescence of Aβ in the absence of any additive increased for 8 h to a final fluorescence of 100. When the monomeric sulfonated GlcNAc (pNP-6S-GlcNAc, **1**) was added to the Aβ solution, no remarkable change was observed in the time-course ThT fluorescence. To improve the weak interaction between 6S-GlcNAc and Aβ, multivalent compounds were then investigated. The addition of glycopolymers of 6S-GlcNAc (**3**–**6**) resulted in inhibition of Aβ aggregation. Interestingly, the inhibition effect was dependent on the sugar content of the polymer. While the glycopolymers with low sugar contents, **5** (28 %) and **6** (12 %), showed strong inhibition of Aβ aggregation, the glycopolymers with high sugar contents, **3** (100 %) and **4** (65 %), did not show strong inhibition. The glycopolymer of GlcNAc without the sulfonate did not change the time-course of ThT fluorescence, and polyacrylamide without the saccharide or sulfonate did not inhibit aggregation.

These data suggest that the sulfonate in the glycopolymer is essential for Aβ aggregation, and that the level of inhibition is also related to the sugar content the glycopolymer. This indicates that the degree of sulfonation in GAGs and their physical properties are important

in the formation of Aβ deposits. It has been reported that GAGs induce and also inhibit the aggregation of Aβ, which is related to the degree of sulfonation and the physical properties.

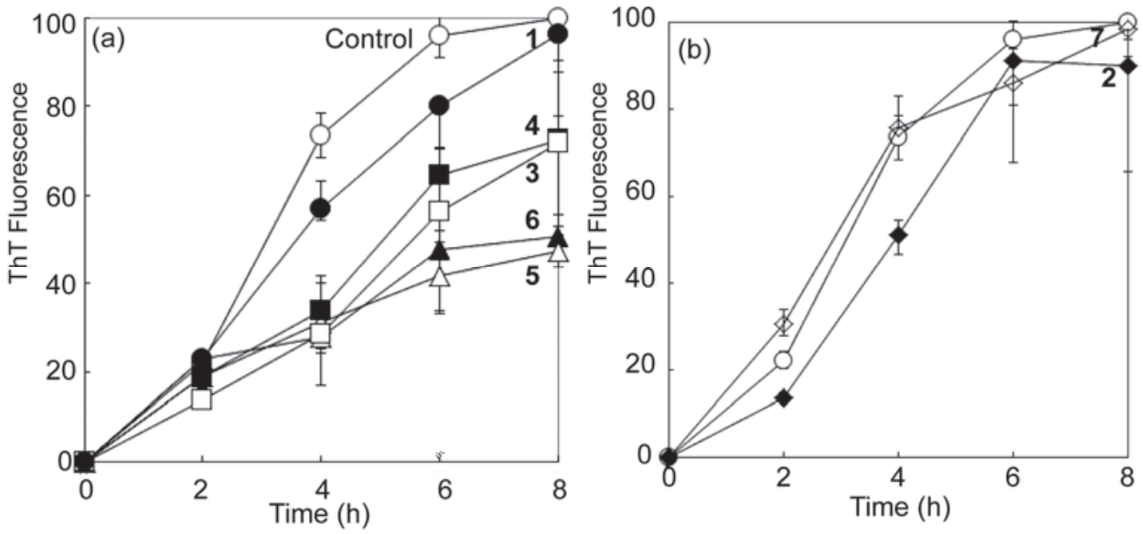


Fig. 3. Time-course of the fluorescence change in ThT at 37°C with Aβ (1-42) and sugar additives (a) with sulfonated GlcNAc of **1**, **3**, **4**, **5**, and **6**, (b) with polymers of **2** and **7**. The concentration of Aβ and sugar was 20 μM and 200mM

2.2 Changes in morphology and neutralization of Aβ by glycopolymers

The morphology of aggregated Aβ was observed in the presence of glycopolymers by AFM (Figure 4). Aβ readily formed amyloid fibrils 15–50 nm wide, 5–15 nm high, and a few micrometers in length. While the addition of monomeric saccharide (**1**) reduced the size of the amyloid fibrils, fibril formation still occurred. The addition of the glycopolymers with 6S-GlcNAc acid (**3–6**) changed the morphology. The addition of the glycopolymer with a low sugar content (**5** and **6**) changed the Aβ into spheres with diameters of 10–250 nm. By contrast, the glycopolymers with a high sugar content (**3** and **4)** did not inhibit fibril formation, and the fibrils increased in size (width 60–300 nm). These morphology observations suggest that Aβ interacted strongly with the sulfonated glycopolymers. These changes were interesting because of the relationship of Aβ's morphology to cytotoxicity (Hoshi et al., 2003, Hardy et al., 2002, Kayed et al., 2003). The cytotoxity Aβ relates the amount of accumulated Aβ, and the morphology, where the oligomeric Aβs with nm order and round morphology has been reported to show the cytotoxity. The results of AFM observation suggest that the development of Alzheimer's disease is related to the degree of sulfonation of GAGs. Control of Aβ's morphology was investigated with specific sulfonated glyco-clusters in a later section.

The neutralization of the cytotoxicity of Aβ was investigated using HeLa cells. When the cells were incubated with Aβ, Aβ reduced the cell survival rate by about 50 %, but the addition of sulfonated glycopolymer (**6**) restored the cell survival rate. Glycopolymer **6** alone did not show any cytotoxicity. The interaction between Aβ and the the glycopolymer ihibitied the accumulation or oligomer formation. Therefore, this glycopolymer, and perhaps GAGs, could safely be used as polymer medicines.

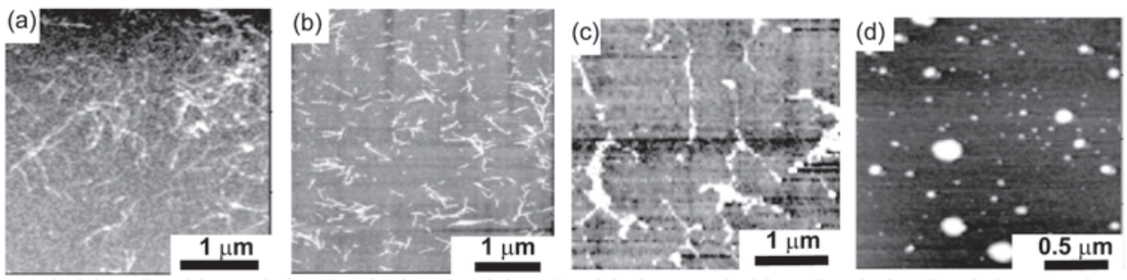


Fig. 4. AFM observations of Aβ(1-42) (a) without glycopolymer, (b) in the presence of **1**, (c) **3** and (d) **6**.

3. The inhibition of amyloid β aggregation by the glycopolymer with precise molecular weight via living radical polymerization

In section 2, glycopolymers with 6S-GlcNAc were shown to bind to Aβ, inhibit Aβ aggregation, and neutralize the cytotoxicity of Aβ. Because of the structural diversity of GAGs, the glycopolymer library is useful to analyze the function of GAGs in Aβ amyloidosis. This could be used to develop a glycopolymer that acts as a better inhibitor of Aβ aggregation. Although it is difficult to analyze the GAGs, the fabrication of glycopolymers is easily achieved by facile radical polymerization. Other polymerization techniques, such as living radical polymerization and graft polymerization, can also be used for this. In this section, a library of glycopolymers with different molecular weights and saccharide structures was prepared to analyze the interaction of Aβ with various glycopolymer. Because natural GAGs are copolymer with hexosamines and uronic acids, the glycopolymers were synthesized using 6S-GlcNAc and glucuronic acid (GlcA).

3.1 Glycopolymer library via living radical polymerization

The glycopolymer library was designed using acrylamide derivatives of 6S-GlcNAc and GlcA (Figure 5). The glycopolymers with 6S-GlcNAc were polyanionic, and were mimics of heparin, while the glycopolymers with GlcA were mimics of hyaluronic acid. The terpolymer of 6S-GlcNAc and GlcA was better mimic of general GAGs in terms of the sugar structure. The sugar contents of the polymers were set at about 10 % based on the results from section 2.1, and the monomer structures were investigated.

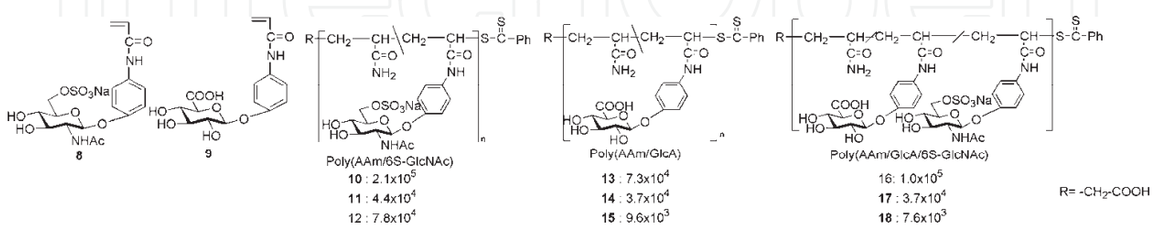


Fig. 5. Chemical structure and molecular weight (M_n) of GAGs mimic polymers in section 3.

Because the molecular weight of GAG affects its biological function, the impact of the molecular weight of the glycopolymer was also investigated. Low molecular GAGs have been reported to show therapeutic effects (Walzer et al., 2002, Zhu et al., 2001) in protein

amyloidosis. Glycopolymers with various molecular weights were synthesized by reversible addition–fragmentation chain transfer (RAFT) living radical polymerization (Moad et al 2008). The living radical polymerization can be used to synthesize well-defined polymers with various molecular weights. Compared with other methods like atom transfer radical polymerization (ATRP), it is easier to synthesize bulky complicated monomers using RAFT living radical polymerization. Dithioesters and trithiocarbonates can be used as reagents in RAFT polymerization. In this study, (thiobenzoyl)thioglycolic acid was used as the RAFT reagent in glycopolymer syntheses.

Glycopolymers with 6S-GlcNAc (**10–12**), GlcA(**13–15**), and terpolymer (**16–18**) were synthesized, and the molecular weights of these polymers were in the order of 10^5 (**10**, **13**, and **16**), 10^4 (**11**, **14** and **17**) and 10^3 (**12**, **15**, and **18**) (Figure 5).

Sample	k_n (s^{-1})	k_e (L mol^{-1} s^{-1})
Aβ (control)	1.3×10^{-6}	3.4
8	1.4×10^{-6}	3.7
10	7.3×10^{-9}	6.6
11	2.5×10^{-8}	5.9
12	5.1×10^{-10}	7.9
9	3.4×10^{-6}	3.0
13	4.2×10^{-6}	2.5
14	2.5×10^{-6}	4.2
15	6.7×10^{-6}	2.5
16	1.3×10^{-6}	4.3
17	6.7×10^{-7}	3.4
18	6.8×10^{-7}	3.1
Heparin	3.1×10^{-6}	3.7
Hyaluronic acid	1.5×10^{-6}	3.4

Table 1. Nucleation and elongation rate constants calculated by fitting ThT data.

The protein Aβ(1-40) was used to analyze the inhibition effect and kinetics. Aβ(1-40) is the main component of amyloid deposits, and has lower ability to aggregate than Aβ(1-42). Because Aβ(1-40) is less likely to aggregate, the amyloid fibril formation was measured with fast shaking (400 rpm) after monomerization by hexafluoro-2-propanol.

3.2 Kinetic analyses of the inhibition of Aβ aggregation in the presence of glycopolymers

Aggregation of Aβ(1-40) was monitored in detail using its ThT fluorescence, which showed a sigmoidal curve (Figure 6). The addition of glycopolymers changed the time-course of ThT fluorescence. The glycopolymers with 6S-GlcNAc (**10–12**) extended the lag phase, which is the time taken for the fluorescence to increase initially, and the final fluorescence intensity was lower than that of control. However, the glycopolymers with GlcA (**13–15**) did not change the lag phase, which suggests these polymers had a weak interaction with Aβ. The ter-glycopolymer with both of 6S-GlcNAc and GlcA showed the best inhibition of Aβ aggregation. The level of inhibition was largely dependent on the sugar structure.

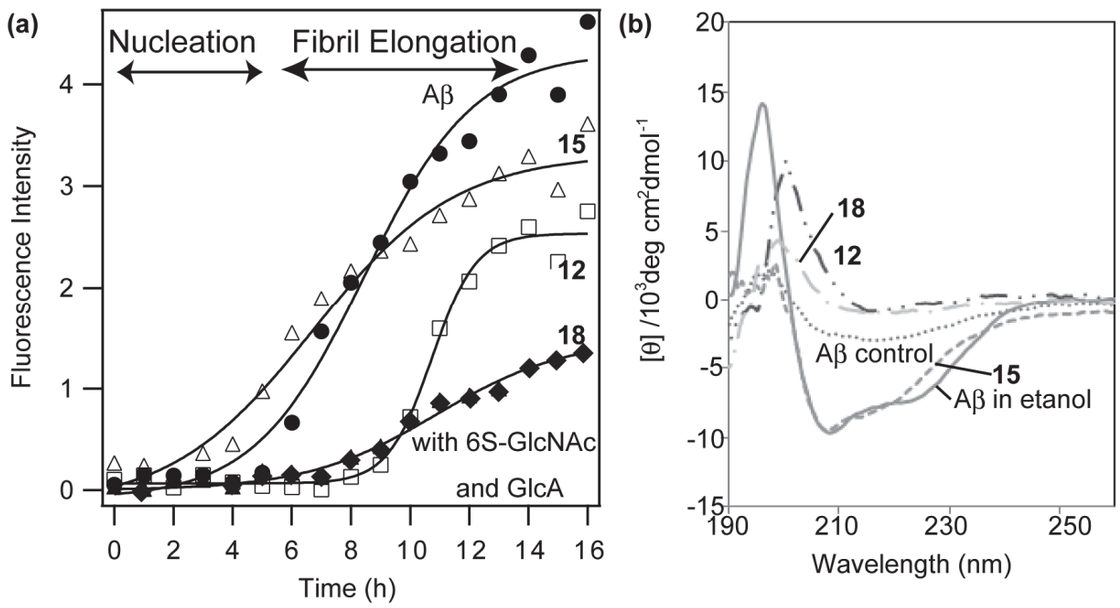


Fig. 6. The time courses of aggregation and the secondary structures of Aβ. (a) Time course of fibril formation with 400 rpm shaking at 37°C. The concentrations of Aβ and sugar were 23 and 200μM. The sugar additives were poly(AAm/6S-GlcNAc)(**12**), poly(AAm/GlcA) (**15**) and poly(AAm/6S-GlcNAc/GlcA) (**18**). (b) The CD spectra of Aβ in the presence of glycopolymers (**12**, **15** and **18**).

The level of inhibition was also dependent on the molecular weight of the polymer. The final fluorescence intensity changed with the molecular weight of the polymer even if the sugar content was the same. Glycopolymers with lower molecular weights produced a lower fluorescence intensity and the longer lag phase than glycopolymers with higher molecular weights.

The aggregation properties were analyzed in detail by fitting of the fluorescence curve. The aggregation of Aβ was analyzed using the processes of nucleation and elongation of the amyloid as follows:

$$nM \rightarrow P_n \qquad \text{(nucleation process, } k_n\text{)}$$

$$M + P_n \rightarrow P_{n+1} \qquad \text{(elongation process, } k_e\text{)}$$

where M and P are the monomeric and polymeric peptides, respectively. The kinetics data are summarized in Table 1. The results were analyzed in terms of the nucleation (k_n) and elongation (k_e) rate constants compared with the control. The glycopolymers with 6S-GlcNAc showed smaller k_n and larger k_e in comparison to the glycopolymers with GlcA, which induced larger k_n and smaller k_e. The 6S-GlcNAc monomer inhibited the nucleation of Aβ, and the GlcA unit inhibited the elongation of the fibril. Among the glycopolymers, the terpolymer with both 6S-GlcNAc and GlcA showed the best inhibition of Aβ aggregation and had the smallest k_n and moderate k_e. The effect of the molecular weight on Aβ aggregation was analyzed using the kinetic parameters. With the 6S-GlcNAc polymer, the polymer with lower molecular weight had a smaller k_n, which gave better inhibition of nucleation. With the

glycopolymer of both 6S-GlcNAc and GlcA, that with the lowest molecular weight was the best inhibitor (**18**). The molecular weight of a polymer affects various physical properties such as mobility, and low molecular weight polymers should have better binding to Aβ. These results were corresponding to those for heparin, which has a low molecular weight.

		19 (monovalent)	**20** (divalent)	**21** (trivalent)
Aggregate Pattern		Fibril	Fibril, Globule	Globule
Fibril	Width Length Height	8-12nm 1-2μm 4-6nm	8-12nm 1-2μm 4-6nm	
Globule	Height Diameter		7-9nm 200-500nm	10-20nm 500-600nm

Table 2. Aggregate patterns and sizes of Aβ (1–42) on **19**, **20** and **21**.

3.3 The conformation and morphology of Aβ aggregation in the presence of various glycopolymers

The AFM results were consistent with the ThT assay. The Aβ aggregates without additives formed amyloid fibrils that were 1–3.5 μm long, 160–230 nm wide, and 15–45 nm high. The Aβ fibrils in the presence of the glycopolymer with 6S-GlcNAc (**12**) were 0.19–2.0 μm long, 110–180 nm wide, and 1–8 nm high. The Aβ fibrils in the presence of the terpolymer (**18**) were 0.10–1.7 μm long, 80–120 nm wide and 1.5-4.0 nm high (Table 2).

The glycopolymer additives induced a specific conformation in Aβ (Figure 7). Aβ(1–40) without additives showed a β-sheet structure with a negative Cotton effect around 220 nm in the CD spectra. The glycopolymer with 6S-GlcNAc gave a β-sheet structure, and the Cotton effect was smaller than for the control Aβ. With the ter-glycopolymers, the spectra showed broad negative Cotton effects with weak intensity. Interestingly, the glycopolymers with GlcA induced a different conformation, with the CD spectra showing negative Cotton effects around 208 nm and 220 nm, which suggests a partial α-helical structure.

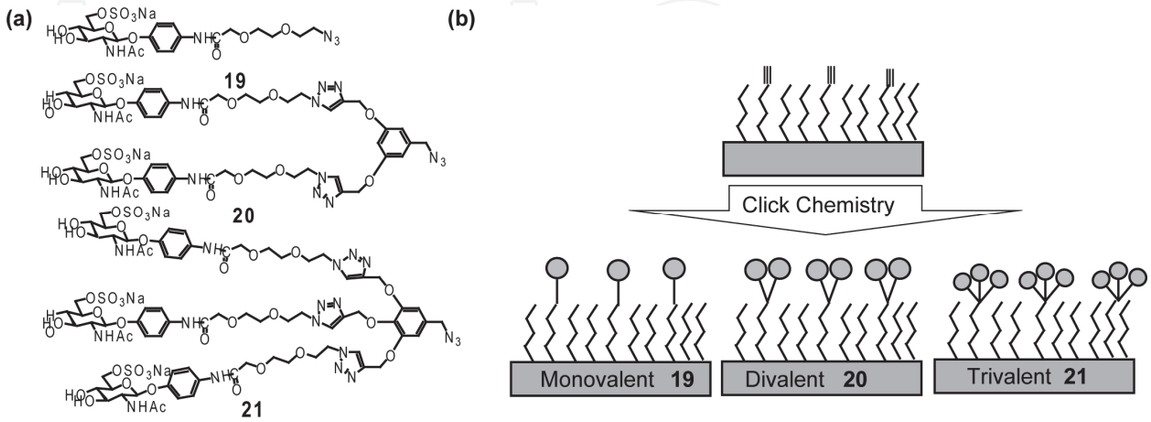


Fig. 7. (a) Chemical structures of glyco-cluster and (b) the schematic illustration of the glyco-cluster at the interface.

The conformation of Aβ was also examined with addition of the natural GAGs heparin (containing 6S-GlcNAc) and hyaluronic acid (containing GlcA). The addition of heparin induced a β-sheet structure with a negative Cotton effect at 218 nm, which was consistent with the CD spectra in the presence of the 6S-GlcNAc glycopolymer. The addition of hyaluronic acid induced a broad negative Cotton effect at 208 nm, which suggests the conformation is not β-sheet. When comparing the sulfonated GAGs like heparin, the roles of uronic acid and hyaluronic acid were not considered because of their weak interactions. If the polyvalent uronic acid or hyaluronic acid can operate as a molecular chaperon in amyloidosis, they will be useful bioactive compounds.

3.4 Mechanism of inhibition of Aβ aggregation

Because the sulfonic acid in the GlcNAc was essential to inhibit Aβ aggregation, this indicates the electrostatic interaction was the driving force behind the inhibition. Both Aβ(1–40) and Aβ(1–42) have a net negative charge, but the proteins of Aβ also contained cationic residues (Arg15, His6, His13, His14, Lys16, and Lys28). The anionic region of 13-HHQK-16 was considered to play an important role, and the HHQK region has been reported to be important in determining the conformation of Aβ (Giulian et al. 1998).

The sugar content of the polymer and the molecular weight effect were interesting for molecular design of an inhibitor. The glycopolymers of 6S-GlcNAc with a high sugar contents did not inhibit Aβ aggregation. The conformation of this glycopolymer was stiff because of the bulky side chain of the polymer, which weakened the interaction with Aβ. The cluster of anionic groups also inhibited the interaction because of electrostatic repulsion that occurred with the net negative charge of Aβ. The role of GlcA was not clear but it provided the hydrogen bonding section and inhibited elongation of the fibrils.

In this section, the effects of the glycopolymer, GAGs, and saccharides were investigated using the glycopolymer technique. The glycopolymer approach clearly showed the role of each saccharide, where sulfonated GlcNAc inhibited nucleation and induced fibril formation and β-sheet formation, and uronic acid induced formation of a partial α-helical structure. The library of glycopolymers with various molecular weights indicated the importance of the physical and chemical properties of GAGs, and suggested low molecular weight polymers were better for inhibition of Aβ aggregation than high molecular weight polymers.

4. The control of Aβ amyloidosis with precise manipulation of the glyco-interface

In section 2 and 3, sulfonated GlcNAc was determined to be a key monomer for Aβ aggregation and protein amyloidosis. The results showed the effect a glycopolymer mimic of a GAG had on the amyloidosis was based on the interaction between Aβ and sulfonated GlcNAc or Aβ and sulfate group. We investigated the control of amyloidosis of Aβ by manipulating the glyco-cluster interface with 6S-GlcNAc. The experiments were conducted on a gold interface for interaction analysis by surface plasmon resonance (SPR), FTIR, and AFM. The protein used was Aβ(1–42), which can have various morphologies because of its self-assembly properties.

4.1 Molecular design of the glyco-interface

Self-assembled monolayers (SAMs) of 6S-GlcNAc were prepared for fabrication of the glyco-cluster interface. An alkyl-disulfide with an acetylenyl group was modified via click chemistry, because an alkyl-disulfide with an oligoethylene glycol has been shown to be bioinert to inhibition of protein adsorption by non-specific interaction (Kolb et al. 2001) (Figure 8). Glyco-clusters with 6S-GlcNAc were synthesized using hydroxyl-benzene templates, and glyco-clusters with mono- (**19**), di- (**20**) and tri- (**21**) valent 6-S-GlcNAc with an azide-group were also synthesized. To modify the substrate, the reactive functional surface was prepared using SAMs. The alkyl-disulfide with an acetylene group was synthesized, and mixed SAMs were prepared using the alkyl-disulfide with acetylene and oligoethylene groups on the gold substrate. The ratio of the acetylene terminal was 20 %. The acetylene group was immobilized by click chemistry.

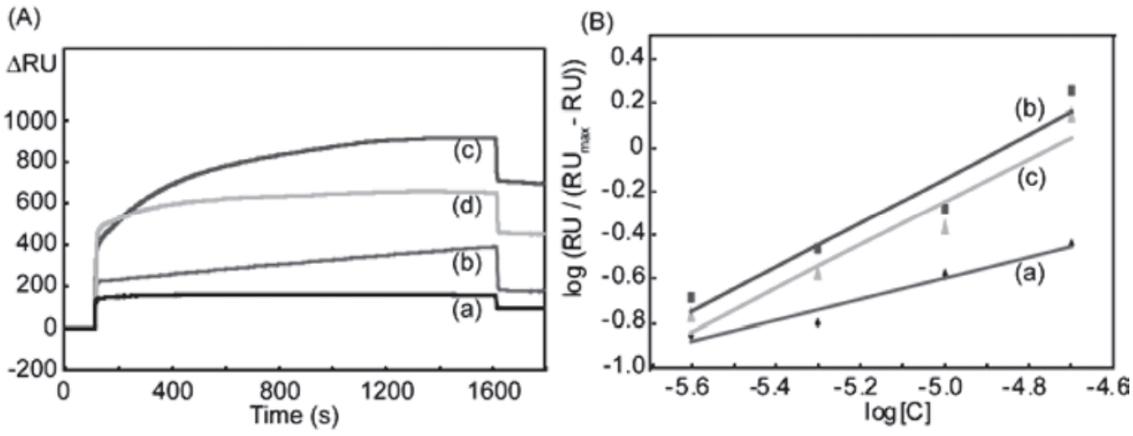


Fig. 8. (A) Time courses of RU change on (a) no-sugar SAM, (b) **19**, (c) **20** and (d) **21**. (B) Hill plot analysis using the RU_{eqmax} on (a) **19**, (b) **20** and (c) **21**.

The glyco-cluster of 6S-GlcNAc was prepared on the gold substrate. Gold substrates have advantages in biological analyses for surface plasmon resonance (SPR), quartz crystal microbalance (QCM), and electrochemistry. The interaction between 6S-GlcNAc and Aβ was analyzed by SPR. The amount of bound Aβ(1–42) (RU_{max}) changed with the valency, and for the different valency glycoclusters was in the order mono- (**19**)<<tri- (**21**)<di-valent (**20**). This suggests that multivalency is important for Aβ binding to the glycocluster. The amount of Aβ bound to the substrate was plotted to calculate the binding constants. The SPR results for the glycoclusters and Aβ were fitted using a Hill plot, not by a Langmuir plot. The multivalent binding was quantitatively analyzed as follows:

$$\log\left(\frac{[RU_{eq\max}]_{C[\mu M]}}{[RU_{eq\max}]_{40[\mu M]}-[RU_{eqmzx}]_{C[\mu M]}}\right)=n\log[C[\mu M]]-n\log K_D$$

where n and K_D represent the Hill coefficient (cooperativity) and apparent binding constant, respectively. The equilibrium constants from the SPR results were 1.49 x 10^{-4}, 1.16 x 10^{-5} and 1.89 x 10^{-5} M for the mono-, di- and tri-valent saccharides, respectively. The Hill coefficients indicated the binding ratios (sugar/Aβ) of the mono-, di-, and tri-valent glycoclusters were 2:1, 1:1 and 1:1, respectively. This shows that multivalent binding is essential even for the monovalent cluster of **19** (binding with an adjacent sugar). The multiple sugar binding was accomplished by the two sugars binding to 13-HHQK-16 or to 13-HHQK-16 and Lys28.

4.2 The morphology of Aβ on the sugar interface

The morphology on the glyco-substrate was investigated by AFM (Figure 9). Interestingly, the morphology of Aβ was dependent on the type of glyco-cluster at the interface. The results are summarized in Table 2. When Aβ(1-42) was incubated on the monovalent 6S-GlcNAc (**19**), fibrils formed that were 4–6 nm high, 8–12 nm wide, and 1–2 μm long. For di-valent 6S-GlcNAc (**20**), both fibrils and spherical aggregates were observed. Furthermore, the incubation of Aβ with trivalent 6S-GlcNAc (**21**) induced formation of only the spherical aggregates with inhomogeneous sizes. The spheres were a mixture of those 10–20 nm and 500–600 nm in diameter.

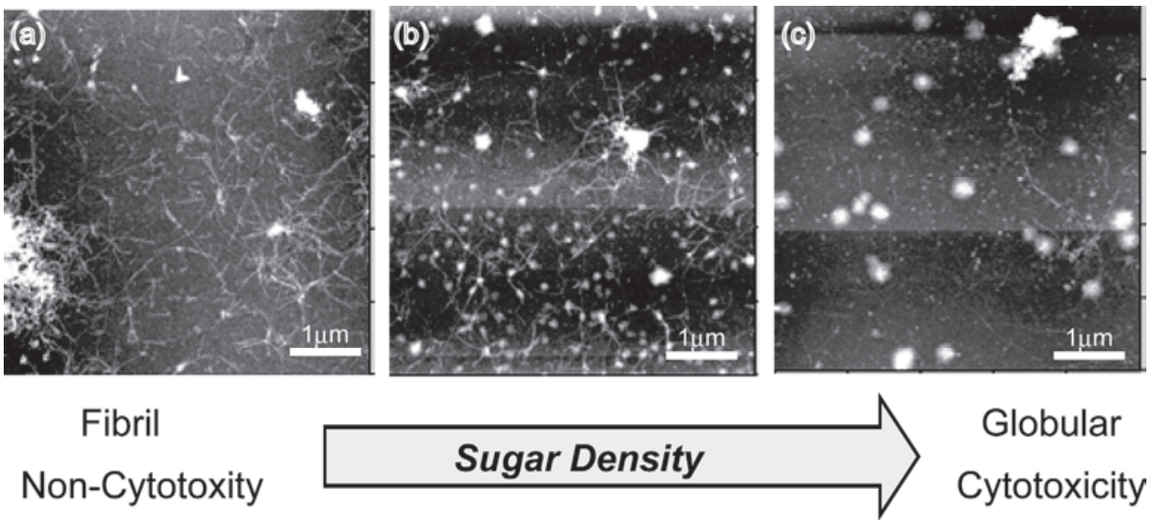


Fig. 9. Morphologies observed by AFM with 6S-GlcNAc glyco-cluster of (a) monovalent (**19**), (b) divalent (**20**) and (c) trivalent (**21**).

The AFM observation of the glycocluster substrate suggested that Aβ(1–42) had a tendency to form fibrils with the low valency 6-S-GlcNAc, and spherical aggregates with higher valency glycoclusters. The densely packed sulfonated or acidic sugars induced a strong morphological change in the Aβ(1-42) aggregates. The morphological change of the amyloid aggregates from fibrils to spheres is consistent with the report of Ban et al.(Ban et al., 2006). The valency of the sulfonated GlcNAc determined the affinity to Aβ, which induced specific morphologies and secondary structures.

The secondary structure of Aβ was measured on the glyco-cluster interface by FTIR in the amide region. The amide I band (C=O stretching) at 1670 cm^{-1} was the main peak in all cases, and corresponded to a β-turn or unknown structure. The band at 1632 cm^{-1} indicated an antiparallel β-sheet structure, which was dependent on the valency of 6S-GlcNAc. Remarkably, that band was the least intense in the spectrum of the trivalent interface (**21**), and the strongest in the spectrum of the monovalent interface (**19**). Taking into account the AFM observations, these results indicated that the fibrils on **19** had predominantly β-sheet structure and the spheres on **21** had little β-sheet structure. The strong binding of Aβ to the 6S-GlcNAc interface suppressed the interaction with each peptide, which reduced the content of β-sheet structure on **21**. The secondary structure revealed that the structure with anti-parallel β-sheet resulted in the fibril formation, and that with less β-sheet structure resulted in the spheres. The negatively charged surface also contributed to the formation of the spherical morphology because of the electrostatic repulsion.

The cytotoxicity of Aβ(1–42) to HeLa cells was evaluated by MTT assay on the substrate. The cell viabilities with Aβ(1–42) on the mono- (**19**), di- (**20**) and tri-(**21**) valent glycoclusters were 104 %, 84 %, 76 %, respectively. These results indicate that the cytotoxicity of Aβ(1–42) varied because of the aggregation process. Aggregation of Aβ(1-42) on the trivalent glycocluster of 6S-GlcNAc formed globular objects, where the small spherical aggregates have been reported to show the high cytotoxity. Though the size of the aggregates were much smaller than that of toxic oligomers (Hoshi et al 2003, Hardy et al 2002, Kayed et al 2003), the observed globular objects on trivalent 6S-GlcNAc had similar round morphology and had the high cytotoxicity. By contrast, aggregation on monovalent 6-S-GlcNAc showed no cytotoxicity. It has been reported that the spherical Aβ exhibit strong cytotoxicity.

The specific morphology of Aβ was induced by the glycocluster on the substrate, which affect the secondary conformation, morphology and finally the cytotoxicity.

5. Conclusion

These studies using artificial glycoclusters revealed the role of sugars in GAGs in amyloidosis. Inhibition and control of amyloidosis of Aβs was accomplished using glycoclusters of glycopolymers and a glycointerface with 6-sulfo-GlcNAc. The glycopolymer with 6-sulfo-GlcNAc inhibited the aggregation of Aβ(1–40) and Aβ(1–42). The appropriate valency of the polymer was essential to aggregation. The glycopolymers of 6-sulfo-GlcNAc with low sugar contents efficiently inhibited the Aβ aggregation and neutralized its cytotoxicity. Kinetic analyses indicated that the sulfonated GlcNAc inhibited the nucleation, and that GlcA of uronic acid inhibited the fibril elongation. The molecular weight of the

polymers was also important, and the glycopolymer with a low molecular weight provided strong inhibition. The low molecular weight glycopolymer with both 6-sulfo-GlcNAc and GlcA showed the strongest inhibition.

With specific glycocluster interfaces, the amyloidosis of Aβ was dependent on the valency and the distance of the 6-sulfo-GlcNAc. High valent 6-sulfo-GlcNAc showed stronger interaction with Aβ than mono valent 6-sulfo-GlcNAc. The dense tri-valent 6-sulfo-GlcNAc induced formation of spheres of Aβ with less β-sheet structure than with the other valencies of 6-sulfo-GlcNAc, and this structure showed cytotoxicity. By contrast, the non-dense monovalent saccharide induced fibril formation with a β-sheet-rich structure without cytotoxicity.

It is difficult to investigate the function of GAGs because of their complex structures, and this method using glycoclusters can be used to clarify their functions. In this investigation, only 6-sulfo-GlcNAc was used as a representative sugar of GAGs. Although wild type GAGs contain various and complicated sulfonated saccharides, this study of GAGs mimics provides useful information on the GAGs functions using precise control of the 6-sulfo-GlcNAc cluster. To extend on this, glycocluster of other sulfonated saccharides are being synthesized in our laboratory.

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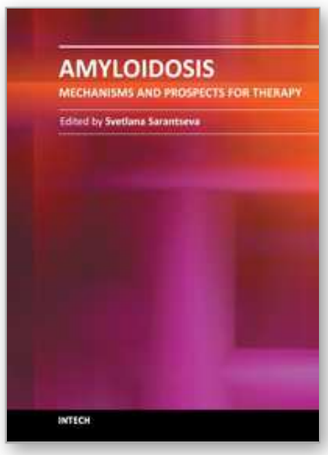
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Amyloidoses are a heterogeneous group of diverse etiology diseases. They are characterized by an endogenous production of abnormal proteins called amyloid proteins, which are not hydrosoluble, form depots in various organs and tissue of animals and humans and cause dysfunctions. Despite many decades of research, the origin of the pathogenesis and the molecular determinants involved in amyloid diseases has remained elusive. At present, there is not an effective treatment to prevent protein misfolding in these amyloid diseases. The aim of this book is to present an overview of different aspects of amyloidoses from basic mechanisms and diagnosis to latest advancements in treatment.

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