Self-Assembly

International Edition: DOI: 10.1002/anie.201501981
German Edition: DOI: 10.1002/ange.201501981

A Self-Assembled Spherical Complex Displaying a Gangliosidic Glycan Cluster Capable of Interacting with Amyloidogenic Proteins**

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Abstract: *Physiological and pathological functions of glycans are promoted through their clustering effects as exemplified by a series of gangliosides, sialylated glycosphingolipids, which serve as acceptors for bacterial toxins and viruses. Furthermore, ganglioside GM1 clusters on neuronal cell membranes specifically interact with amyloidogenic proteins, triggering their conformational transitions and leading to neurodegeneration. Here we develop a self-assembled spherical complex that displays a cluster of the GM1 pentasaccharide, and successfully demonstrate its ability to interact with amyloid β and α-synuclein. Due to the lack of hydrophobic lipid moieties, which would stably trap these cohesive proteins or give rise to toxic aggregates, this artificial cluster enabled NMR spectroscopic characterization of the early encounter stage of protein interactions with its outer carbohydrate moieties, which were not observable with previous glycan clusters.*

Oligosaccharides play pivotal roles in physiological and pathological contexts. Oligosaccharide functions are often promoted when they form clusters on cellular membranes; as exemplified by gangliosides, a group of glycosphingolipids containing sialic acid, which mediate cell adhesion and signal transduction and provide acceptor sites for microbial toxins and viruses.[1] Hence, ganglioside clusters are potential medicinal and therapeutic targets.[2] Particular attention has been paid to ganglioside GM1, which is known to serve as a receptor for cholera toxin, polyomavirus, and autoantibodies associated with the Guillain–Barré syndrome.[3–5] These interactions are characterized by their multivalent binding to GM1 clusters. Therefore, artificial clusters of the GM1 pentasaccharide moiety could be designed and created for detecting, capturing, and adsorbing toxic entities. Recently, accumulating evidence has indicated that GM1 clusters on neuronal cell membranes specifically interact with amyloid β-protein (Aβ), triggering its conformational transition into toxic aggregates responsible for the onset and development of Alzheimer's disease.[6] Pathological interactions of ganglioside clusters have also been proposed for other amyloidogenic proteins, including α-synuclein and prion protein, which are associated with Parkinson's disease and Creutzfeldt-Jakob syndrome, respectively.[7–9] The ganglioside binding of these proteins is not described as lectin-like stoichiometric binding, although they exhibit certain specificities for the outer carbohydrate moieties. Rather, ganglioside clusters offer catalytic environments for conformational alteration of the proteins coupled with their transient, multistep binding.[2,6b] To characterize the underlying mechanisms of these pathological molecular processes, artificial ganglioside clusters have been prepared based on micelles and bicelles, primarily for solution-phase NMR spectroscopic analyses.[10,11] These studies indicated that the size and curvature of the ganglioside clusters are determining factors in the interactions and subsequent aggregate formation, highlighting the need for development of gangliosidic carbohydrate assemblies that are systematically controllable in terms of these factors. These previous studies also revealed that the hydrophobic environment provided by the lipid hydrocarbon chains is responsible for the formation and accommodation of α-helical segments of Aβ and α-synuclein.[10a,c,12] Furthermore, the hydrophilic/hydrophobic interface facilitates amyloid formation of these pathogenic proteins. These findings suggest that creation of oligosaccharide clusters lacking the ceramide moiety will open up new possibilities for characterizing the early stages of interactions of ganglioside clusters with amyloidogenic proteins.

Here, we created well-defined GM1 clusters on the periphery of an $M_{12}L_{24}$ spherical complex,[13] a large scaffold assembled from 12 Pd^{2+} ions (M) and 24 bent ligands (L) that define a stable discrete interface.[13c] An NMR spectroscopic study revealed that the initial encounter complex formation between the GM1 pentasaccharide cluster and Aβ occurs at the N-terminal segment of Aβ. This approach was also successfully applied to the observation of the interaction between the GM1 pentasaccharide cluster and α-synuclein.

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[**] This research was supported by JST, ACCEL, and KAKENHI for Young Scientists (A) (24685010), for Scientific Research on Innovative Areas (25102007, 25102001, and 25102008), for Young Scientists (B) (15K21680), and for Challenging Exploratory Research (26560451), the Research Funding for Longevity Sciences (25-19) from National Center for Geriatrics and Gerontology, the Okazaki ORION project, and the Nanotechnology Network Project and Nanotechnology Platform Program at IMS.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201501981.

GM1 pentasaccharide clusters were constructed by self-assembly of Pd^{2+} ions with bidentate ligand **1**, which bear the sugar moiety of ganglioside GM1 at the apex of the ligand. Since the commercially available GM1 source is expensive and limited, an efficient and short synthetic route to introduce the GM1 sugar onto the ligand was explored (Figure 1). The reductive amination reaction was performed by addition of $NaBH_3CN$ (1.0 equiv) to a solution of lyso-GM1 **2** (1–5 mg scale) and bidentate ligand **3** (5.0 equiv), which bears an aldehyde group at the apex, in hexafluoro-2-propanol, to afford GM1-anchored ligand **4**. The lipid chain was removed by treatment of crude product **4** in a mixed solvent system of $MeOH/CH_2Cl_2$ (1:1) with ozone (4.0 equiv), followed by addition of $NaBH_4$ (10 equiv), affording ligand **1** in 67% yield from lyso-GM1 **2** (Figure 1B). Structural determination of **1** was performed by spectroscopic analyses and high-resolution mass spectrometry.

GM1 sugar cluster **5**, which accumulates just 24 GM1 sugar moieties on an $M_{12}L_{24}$ spherical complex, was self-assembled from the ligand-bearing GM1 sugar **1** (1.91 μmol) in DMSO (89.3 μL). This was treated with $Ca(NO_3)_2$ (46.7 μmol) in D_2O (6.34 μL) at 50 °C for 10 min, followed by addition of $Pd(NO_3)_2$ (1.15 μmol) in DMSO at 50 °C for 12 h (Figure 1A). The initial addition of $Ca(NO_3)_2$ was essential for forming the spherical complex, presumably because the weak interaction between the Ca^{2+} ions and sugar moieties prevented trapping of the Pd^{2+} ions at the sugar moieties. Dialysis of the solution of sphere **5** in DMSO against H_2O removed the DMSO and $Ca(NO_3)_2$, giving a solution of sphere **5** in H_2O. The ^1H NMR spectra of ligand **4** and sphere **5** show a downfield shift for the ^1H NMR signals at the pyridyl α-positions, which indicates the formation of coordination bonds between the pyridyl groups and Pd^{2+} ions (Figure 2). The ^1H signals of sphere **5** are broad due to the large size of the structure. The number of signals did not change between ligand **4** and sphere **5**, suggesting the formation of a highly symmetric structure. The diffusion coefficient (D) of sphere **5** was determined by DOSY measurements; the obtained value, $D = 6.3 \times 10^{-11}\ m^2\,s^{-1}$, is consistent with a diameter of 5.7 nm, which corresponds to the value obtained for the structure simulated through molecular mechanics (MM) calculations.[14]

The possible interaction between sugar cluster **5**, bearing an artificial GM1 cluster without hydrophobic ceramide moieties, and Aβ was examined to characterize their encountering state. A solution of uniformly ^{15}N-labeled Aβ-40 with 40 amino acid residues (240 μL, 0.11 mM, 25 nmol) in aqueous phosphate buffer (30 mM) was added to a solution of sphere **5** (360 μL, 0.070 mM, 25 nmol) in aqueous phosphate buffer (30 mM, pH 6.8) at 27 °C. Under these conditions, 1.0 equiv of sphere **5** bearing 24 pentasaccharides was used for every 1.0 equiv of Aβ-40. The solution was examined by ^1H–^{15}N HSQC NMR spectroscopy to observe the amide signals of Aβ-40. When we compared the signal intensities in the ^1H–^{15}N HSQC spectra for Aβ-40 with and without sugar cluster **5**, all signals were attenuated. Intriguingly, the signals originating from the N-terminal segment became undetectable due to extreme line broadening (Figure 3A, dark blue). Although the NH signal intensities were recovered upon reduction of the amount of **5** used (2.5 nmol, 0.10 equiv), the N-terminal signals of Aβ-40 remained significantly attenuated (Fig-

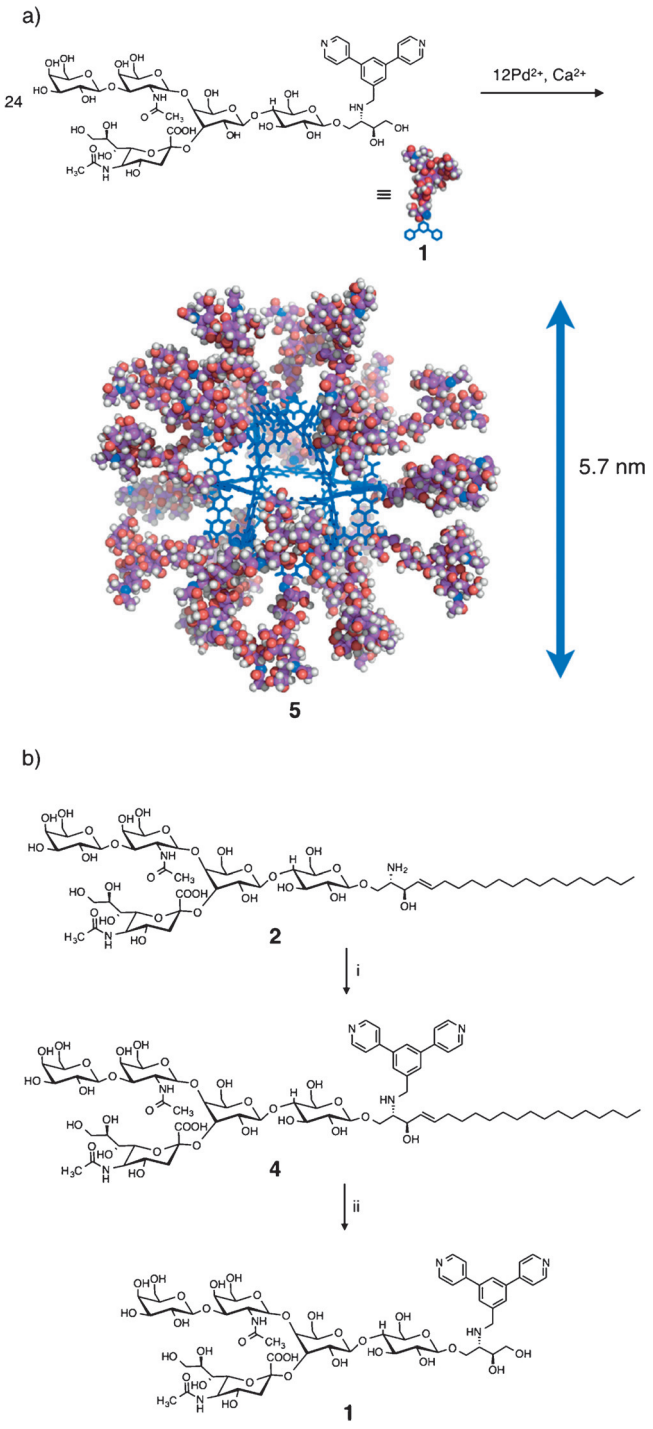

Figure 1. A) Synthesis of sugar cluster **5** from Pd^{2+} ions and bidentate ligand **1** bearing GM1 sugar. The molecular structure of sphere **5** was optimized by MM calculations (Pd: yellow, C: light blue, N: blue, O: red, H: gray). B) Synthesis of ligand **1** by the ligand-tethering reaction i [conditions: 1) 2,3-bis(4-pyridyl)benzaldehyde **3**, HFIP, 50 °C, 1 h; 2) $NaBH_3CN$, $HFIP/CH_3OH$ (15:1), 50 °C, 2 h] and the lipid chain cleavage reaction ii [conditions: 1) O_3, CH_2Cl_2/CH_3OH (1:1), −80 °C, 3–6 h; 2) $NaBH_4$, CH_2Cl_2/CH_3OH (1:1), −80 °C to RT, 2 h]. The overall yield from GM1 **2** was 67%.

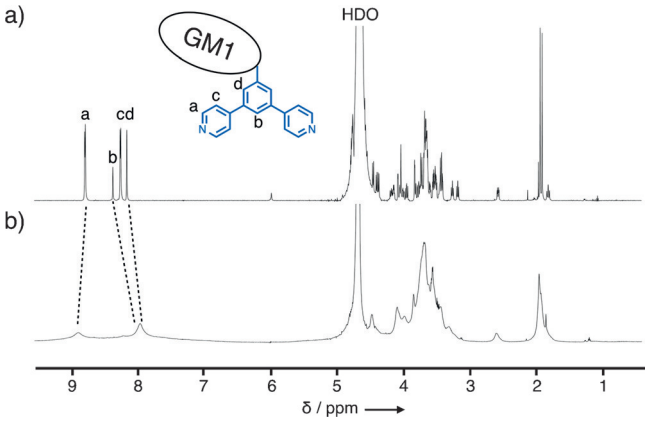

Figure 2. ^{1}H NMR spectra (600 MHz, 27 °C, D_2O) of A) ligand **1** and B) complex **5**.

ure 3 A, light blue). Effective interactions were observed in the range of 0.10 to 1.0 equiv of sugar cluster **5**, with signal decays for all amino acid residues. The specific attenuation of the N-terminal signals was presumably due to selective involvement of the N-terminal segment of Aβ-40 in the interaction with the GM1 pentasaccharide cluster on sphere **5**. This is characterized as a dynamic process in the intermediate regime, whereas the C-terminal segment is scarcely involved in the interaction.

For Aβ-28,[15] which has 28 amino acid residues and lacks the C-terminal residues 29–40, 1.0 equiv of sugar cluster **5** was added in the same manner (Figure 3 B). The truncated Aβ-28 showed a larger magnitude of signal decay than Aβ-40 over all amino acid residues, which again underscored the selective involvement of the N-terminal segment, confirming the results obtained for Aβ-40. This was presumably due to the decrease in size of the noninteracting part of the protein. As control experiments, ^{1}H–^{15}N HSQC NMR spectra of Aβ-40 (25 nmol) with monomeric GM1 pentasaccharide (25 nmol) or $Pd(NO_3)_2$ (2.5 nmol) were measured. In both cases, signal decay was negligible, demonstrating that cluster formation of the GM1 pentasaccharide is a prerequisite for its specific interaction with Aβ-40 (see Figures S9 and S10 in the Supporting Information). Inspection of all data suggests that Aβ could form an encounter complex specifically with the sugar clusters of GM1 gangliosides through its N-terminal region. This observation is in marked contrast to the topological mode of Aβ-40 lying on the hydrophilic/hydrophobic interface of GM1 micelles, in which the two α-helices (His^{14}-Val^{24} and Ile^{31}-Val^{36}) and the C-terminal dipeptide segment (Val^{39}-Val^{40}) are in contact with the hydrophobic interior of the ganglioside clusters.[10a]

Brain deposition of hereditary Aβ variants with single amino acid substitutions crucially depends on the presence of favorable gangliosides.[16] For example, Dutch-, Italian-, and Iowa-type Aβ variants require GM3 ganglioside for their assembly, whereas GD3 is upregulated at the site of Flemish-type Aβ deposition. These data suggest that the outer carbohydrate structures displayed on neural cells are critical determining factors for specificities of the interactions between the hereditary Aβ variants and the various gangli-

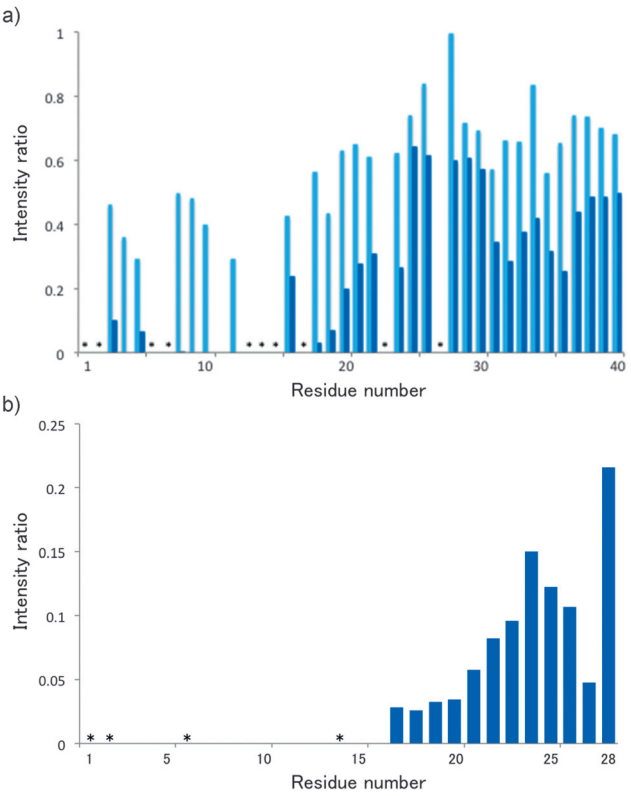

Figure 3. A) Relative intensities of backbone NH signals of ^{15}N-labeled Aβ-40 protein plotted against the amino acid sequence. The applied amount of sugar cluster **5** was either 1.0 equiv (dark blue) or 0.10 equiv (light blue). The signals were observed from ^{1}H–^{15}N HSQC NMR spectra (600 MHz, 27 °C, 30 mM aqueous phosphate buffer, pH 6.8, H_2O/D_2O (90:10)). Intensity ratio was obtained by dividing each peak height value of Aβ-40 protein in the presence of sugar cluster **5** by that of this protein alone. Asterisks indicate amino acid residues that did not exhibit observable peaks in the spectrum due to severe broadening. B) Relative intensities of backbone NH signals of ^{15}N-labeled Aβ-28 protein plotted against the amino acid sequence. The applied amount of sugar cluster **5** was 1.0 equiv. The signals were observed from ^{1}H–^{15}N HSQC NMR spectra (600 MHz, 27 °C, 30 mM aqueous phosphate buffer, pH 6.8, H_2O/D_2O (90:10)). Intensity ratio was obtained by dividing each peak height value of Aβ-28 protein in the presence of sugar cluster **5** by that of this protein alone. Asterisks indicate amino acid residues that did not exhibit observable peaks in the spectrum due to severe broadening.

osides. Our self-assembling cluster will be applicable for displaying other gangliosidic oligosaccharides, opening the door to studies on the structural mechanisms underlying such pathological processes.

To examine the applicability of artificial gangliosidic sugar cluster **5**, without a hydrophobic lipid chain for identifying the carbohydrate interacting sites in other amyloidogenic proteins, α-synuclein was subjected to NMR spectroscopic analysis under the same conditions using 1.0 equiv of sugar cluster **5**. Because α-synuclein is a large, intrinsically unstructured protein composed of 140 amino acid residues, we performed ultrahigh-resolution NMR spectroscopic experiments with a 920 MHz spectrometer.[17] Analysis of the ^{1}H–^{15}N HSQC NMR spectra revealed reduction in peak intensity over all amino acid residues of α-synuclein, with marked

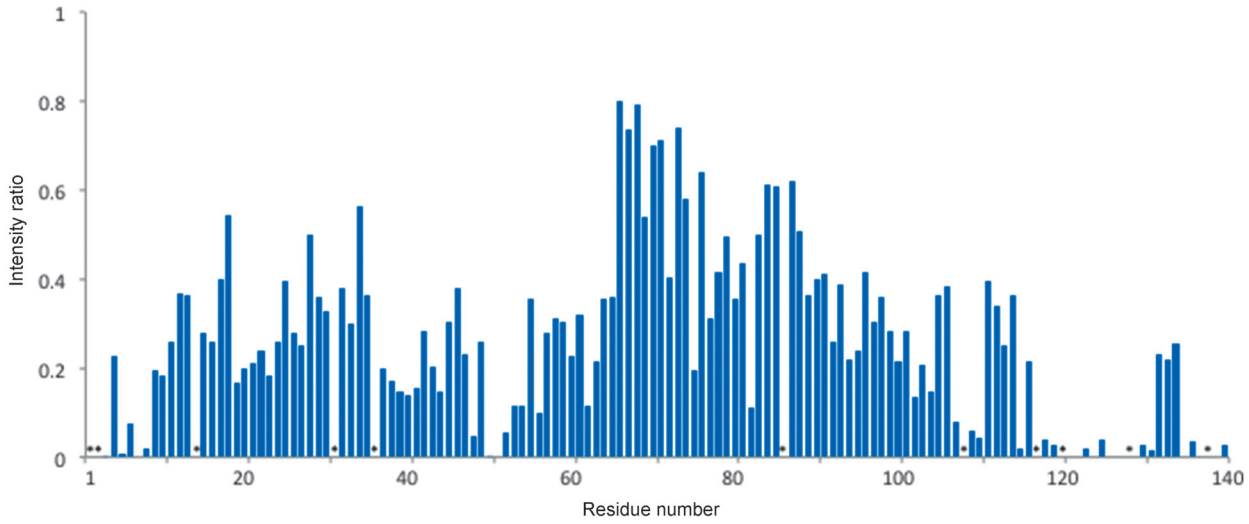

Figure 4. Relative intensities of backbone NH signals of ^{15}N-labeled α-synuclein protein plotted against the amino acid sequence. The applied amount of sugar cluster **5** was 1.0 equiv. The signals were obtained from ^{1}H–^{15}N HSQC NMR spectra (920 MHz, 10°C, 15 mM aqueous phosphate buffer, pH 6.8, H_2O/D_2O (90:10)). Intensity ratio was obtained by dividing each peak height value of α-synuclein in the presence of sugar cluster **5** by that of this protein alone. Asterisks indicate proline residues or amino acid residues that did not exhibit observable peaks in the spectrum due to severe broadening.

attenuation in its N- and C-terminal segments, suggesting these regions participate in the interaction with the GM1 pentasaccharide cluster (Figure 4). Our previous NMR spectroscopic analysis using GM1-embedding small bicelles suggested that the N-terminal segment of α-synuclein is involved, as the most "ganglioside-philic" region, in the membrane-landing process on ganglioside clusters.[11c] This is most likely followed by membrane-anchoring α-helix formation, as observed with anionic vesicles.[12] Artificial sugar cluster **5** presumably exhibits a large continuous surface covered by GM1 pentasaccharides, as compared with the GM1-embedding small bicelles, and lacks the hydrophobic interior required for accommodation of the α-helical segments of α-synuclein. This enables us to identify the previously unidentified secondary ganglioside binding site, that is, the C-terminal segment, thereby offering a new tool to provide us with information on the interaction mode complementarily with micelle- and bicelle-based systems.

In summary, a well-defined sugar cluster was constructed through the modification of a self-assembled $M_{12}L_{24}$ sphere with the sugar moiety of natural bioactive ganglioside GM1. The spherical complex showed recognition abilities toward amyloidogenic proteins Aβ and α-synuclein at their specific sites, which were promoted by oligosaccharide clustering without the use of hydrophobic lipid chains. These chains would otherwise stably trap the cohesive proteins, precluding the observation of early encounter interactions or give rise to toxic aggregates. Further variation of ganglioside clusters with different size and curvature would be accessible for systematic studies using a series of self-assembled spherical complexes as the scaffolds.[18] Our novel artificial ganglioside–glycan cluster will be useful not only as a novel analytical tool, but also as a prototype of self-assembled sorbent targeting the ganglioside-interacting hazardous substances used in therapeutic and diagnostic applications, offering new possibilities for developing bioprobes, biosensors, and drug delivery systems.

Keywords: amyloid β · NMR spectroscopy · self-assembly · sugar cluster · α-synuclein

How to cite: *Angew. Chem. Int. Ed.* **2015**, *54*, 8435–8439
Angew. Chem. **2015**, *127*, 8555–8559

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Received: March 3, 2015
Revised: March 31, 2015
Published online: May 26, 2015

