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Specific surface modification of the acetylene-linked glycolipid vesicle by click chemistry†

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A novel glycolipid with a terminal acetylene was synthesized and used to prepare unilamellar vesicles. Using these vesicles, a convenient method was developed for the specific modification of the vesicle surface using the photoresponsive copper complex [Cu(OH$_2$)(cage)] as the catalyst for a click reaction.

The biological membrane provides many functions necessary for biological activities, including acquisition of nutrients and energy sources, reproduction, cellular recognition. Most of these functions are provided by membrane proteins and carbohydrates on the lipid bilayer surface. Additionally, these functional groups are specifically located either on the inner or outer surfaces of the lipid bilayer. Recently, functionalization of vesicles *via* modification with various functional ligands, including biological molecules, has attracted significant interest.[1–3] These studies require an efficient and convenient method for the introduction of functional ligands onto the surfaces of vesicles.[4,5] There are many reports on the outer surface modification of vesicles; however, there have not yet been any reports on the specific modification of the inner surfaces of vesicles. A specific modification of both the inner and outer surfaces of vesicles with different functional ligands is of importance for the preparation of artificial biological membranes, because the inner and outer surfaces of biological membranes are asymmetric due to the spatial organization of their constituent lipids and proteins.

Herein, we report a method for the specific modification of the inner or outer surfaces of a glycolipid vesicle using click chemistry. Glycolipid is one of the major compounds of cellular membranes and comprises 90% of thylakoid membrane, where photosynthetic reactions occur.[6] However, relatively little research has been focused on vesicles using glycolipids as compared to those using phospholipids. We therefore synthesized a novel glycolipid with a terminal acetylene in a carbohydrate moiety, and prepared unilamellar vesicles with this synthesized glycolipid. The vesicle has an acetylene moiety of the synthesized glycolipid on both the inner and outer surfaces. This terminal acetylene moiety allows for conjugation with azido compounds *via* a copper(I) catalyzed 3+2 cycloaddition.[7] This reaction is known as "click chemistry", which is a powerful tool for biological applications, because of the high efficiency of the reaction even in water, and unreactive nature of both azides and alkynes toward the functional groups present in biomolecules.[8] The modification of either the inner or outer surfaces of the vesicles was achieved by controlling the click chemistry. In the case of inner surface modification of the vesicles, the photo-responsible copper caged compound [Cu(OH$_2$)(cage)] was used with UV irradiation after vesicle encapsulation (Fig. 1).

Glycolipid **1** was synthesized by linking an acetylene-linked sugar with a monosaccharide glycolipid (Scheme 1). The acetylene-linked sugar was synthesized by reacting a D-galactopyranose derivative

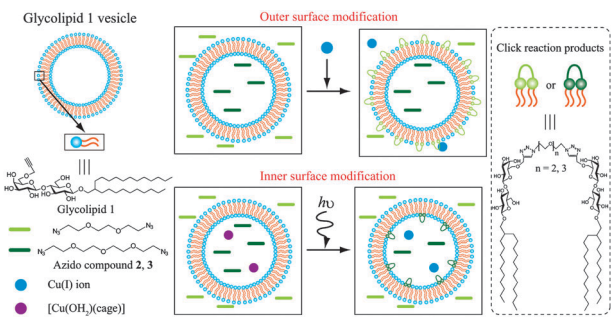
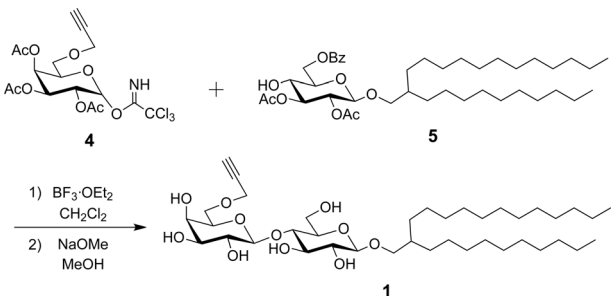


Fig. 1 Schematic illustration of the specific surface modification of an acetylene-linked glycolipid vesicle using click chemistry.



Scheme 1 Synthesis of the glycolipid **1**. The acetylene-linked sugar head group is linked to the tail group through a β-*o*-glycosidic bond.

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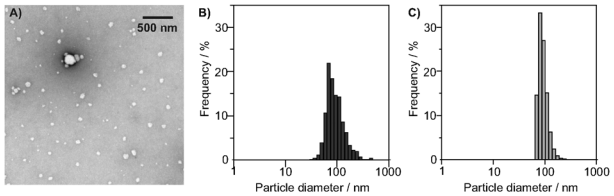

Fig. 2 (A) Transmission electron microscopy image of the glycolipid **1** vesicles (0.1 mM) stained with uranyl acetate. (B) Particle size histogram of the glycolipid **1** vesicles (0.1 mM) by TEM. (C) Histogram analysis of the DLS measurements of the glycolipid **1** vesicles (0.1 mM) in water. The solution was filtered through a 0.45 μm syringe filter (Toyo Roshi, Japan) prior to use, and then the DLS measurement of the sample was performed at a fixed scattering angle of 90° at 25 °C.

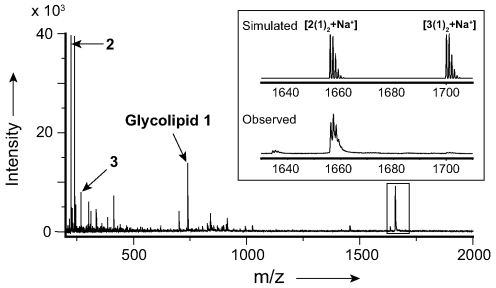

Fig. 3 ESI-MS spectra of the glycolipid **1** vesicle encapsulating azido compound **3** after addition of azido compound **2**, $CuSO_4 \cdot 5H_2O$, and sodium ascorbate at room temperature.

with propargyl bromide.[9] The monosaccharide glycolipid was synthesized from D-glucose and 2-decyl-1-tetradecanol.[10] Unilamellar vesicles of glycolipid **1** were prepared by extrusion of the vesicles through a 0.45 μm syringe filter after sonication, according to the methods reported previously.[11,12] The size of the glycolipid **1** vesicles was measured by transmission electron microscopy (TEM) and dynamic light scattering (DLS). The TEM image of the vesicles showed circles with a diameter of approximately 100 nm, indicating the formation of vesicles using glycolipid **1**. DLS measurement showed that the average particle size of the vesicles in water was 93.8 ± 21.6 nm. These observations suggest the formation of unilamellar vesicles (Fig. 2).

The modification of the glycolipid **1** vesicle surface by click chemistry was carried out using two azido compounds (**2**, **3**) based on oligo (ethylene glycol) with different molecular weights.[13] To modify the outer surface of the vesicles, the copper(I) catalyst and azido compound **2** were added to a vesicle solution. When the resulting mixture was measured by electrospray ionization mass spectrometry, the molecular weights of glycolipid **1** and the click reaction product were observed (Fig. S1, ESI†). The average particle size of the glycolipid **1** vesicle after surface modification was 82.7 ± 16.1 nm as determined by DLS and TEM measurements (Fig. S2 and S3, ESI†), indicating that the particle size did not change by the surface modification. These results indicate that the vesicles maintain their structure by the modification with the azido compound **2** using the click reaction, and the outer surface of the vesicle may be selectively modified.

In order to confirm the specific modification of the vesicle surface, a vesicle solution containing two azido compounds, one located in the internal aqueous phase and the other one outside of the vesicle, was prepared, and modification of the outer surface was carried out by adding the copper(I) catalyst to the solution outside of the vesicle.

The vesicles encapsulating the azido compound were obtained by dispersing a dried glycolipid **1** thin film in water (1 mM) containing azido compound **3** (5 mM) with vortexing, followed by sonication. The resulting vesicles were then frozen in liquid N_2 and thawed. This freeze–thaw cycle was repeated at least three times. Excess azido compound **3** in the solution was removed by gel filtration chromatography using a HiTrap Desalting column (GE Healthcare). The outer surface modification of the vesicle encapsulating azido compound **3** was then carried out in the presence of 0.1 mM azido compound **2**, 0.02 mM copper sulfate, and 0.05 mM sodium ascorbate, which were in the solution outside of the vesicle, overnight. ESI-MS measurement of this resulting mixture showed the signal for the molecular weight of the click reaction product of glycolipid **1** and azido compound **2**, while the product of glycolipid **1** and azido compound **3** was not observed. This result indicates that the selective modification of the outer surface of the glycolipid **1** vesicle can be achieved using this method. Furthermore, this result also suggests that the azido compounds and the copper ions do not translocate across the bilayer membrane of glycolipid **1** at room temperature (Fig. 3).

To carry out the selective modification of the inner surface of the vesicles, it is necessary to perform the click reaction after formation of the vesicle. If a free copper ion is added to the solution during vesicle formation, the click reaction could occur before formation of the vesicle. To solve this problem, the photoresponsive copper complex [$Cu(OH_2)$(cage)], which releases a copper ion by ultraviolet (UV) light irradiation, was used.[14] When using [$Cu(OH_2)$(cage)] instead of a free copper ion, the click reaction does not occur in the dark. Photolysis with 350 nm UV light cleaves the ligand backbone of [$Cu(OH_2)$(cage)], releasing copper ions so that the click reaction can proceed.

A vesicle thus prepared using 1 mM glycolipid **1**, 5 mM azido compound **3**, 2 mM [$Cu(OH_2)$(cage)], and 0.5 mM sodium ascorbate contains [$Cu(OH_2)$(cage)], azido compound **3**, and sodium ascorbate in the internal aqueous phase. The azido compound **2** was then added to this vesicle solution to give a final concentration of 0.02 mM. The resulting vesicle solution contained azido compound **2** in the solution outside of the vesicle, and azido compound **3** in the inside of the vesicle. [$Cu(OH_2)$(cage)] and ascorbate were also located inside the vesicle, so the click reaction could be initiated by irradiation with UV light. After irradiation for one hour, the modification by click reaction was carried out overnight at room temperature in the dark. ESI-MS measurement of the resulting mixture showed the signal for the molecular weight of the click reaction product of glycolipid **1** and azido compound **3**, but not for that of glycolipid **1** and azido compound **2**. This result suggests that the inner surface of the vesicle was selectively modified (Fig. 4).

In summary, we have synthesized a novel glycolipid with a terminal acetylene and prepared unilamellar vesicles from it. A convenient method for the specific inner surface modification of the vesicle was developed using the photoresponsive copper complex [$Cu(OH_2)$(cage)] as the catalyst for a click reaction. The inner surface modification is achieved *via* the encapsulation

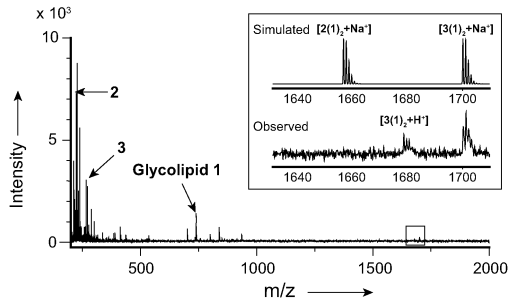

Fig. 4 ESI-MS spectra of the glycolipid **1** vesicle encapsulating azido compound **3**, [Cu(OH$_2$)(cage)], and sodium ascorbate after addition of azido compound **2** and irradiation of UV light for one hour at room temperature.

of the photoresponsive copper complex, ascorbate, and azido compound followed by the UV light irradiation. This technique can be applied to the functionalization of both the inner and outer vesicle surfaces. The click reaction is a versatile tool for biological applications, and the specific surface modification of vesicles as described in this report can be applied after reconstitution of membrane proteins in the vesicles. This approach will permit the effective functionalization of vesicle surfaces for biological applications.

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