Agglutination of Human Polyomaviruses by Using a Tetravalent Glycocluster as a Cross-Linker

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ABSTRACT: Two kinds of tetravalent double-headed sialo-glycosides with short/long spacers between the Neu5Acα2,6Galβ1,4GlcNAc unit and ethylene glycol tetracetic acid (EGTA) scaffold were found to be capable of binding to virus-like particles of Merkel cell polyomavirus (MCPyV-LP). The binding process and time course of interaction between the tetravalent ligand and MCPyV-LP were assessed by dynamic light scattering (DLS). On the addition of increasing concentrations of ligand to MCPyV-LP, larger cross-linked aggregates formed until a maximum size was reached. The binding was stronger for the tetravalent ligand with a short spacer than for that with a long spacer. The binding of the former ligand to the virus was observed to proceed in two stages during agglutination. The first step was the spontaneous formation of small aggregates comprising the cross-linked interaction. In the second step, the aggregates grew successively larger by cooperative binding among the initially produced small aggregates. In transmission electron microscopy, the resulting complex was observed to form aggregates in which the ligands were closely packed with the virus particles. The cross-linked interaction was further confirmed by a simple membrane filtration assay in which the virus-like particles were retained on the membrane when complexed with a ligand. The assay also showed the effective capture of particles of pathogenic, infectious human polyomavirus JCPyV when complexed with a ligand, suggesting its possible application as a method for trapping viruses by filtration under conditions of virus aggregation. Collectively, these results show that the tetravalent glycocluster serves as a ligand not only for agglutinating MCPyV-LP but also for trapping the pathogenic virus.

INTRODUCTION

The saccharide units play an important role in viral adhesion and infection. So far, the common strategy to study carbohydrate–viral carbohydrate-binding protein and carbohydrate–virus interactions is to present carbohydrate ligands on various macro- or mid-molecule scaffolds such as polymers, polypeptides, polysaccharides, dendrimers, particles, and others. In particular, a large variety of sialylated scaffolds have been shown mainly to not only bind to influenza virus but also to a far lesser extent to other viruses known to interact with sialylated glycans such as human polyomaviruses.

In general, human polyomaviruses are ubiquitous and cause asymptomatic infections. However, some polyomaviruses can cause serious illnesses, especially in immunocompromised individuals. As an example, Merkel cell polyomavirus (MCPyV) causes a skin cancer named Merkel cell carcinoma. In addition, JCPyV, the first identified human polyomavirus, is well characterized as the etiologic agent of progressive multifocal leukoencephalopathy. The particles of a polyomavirus such as MCPyV consist of 72 capsomeres, which mainly comprise a major capsid viral protein (VP1) with binding sites for sialylated glycans. A structural analysis has shown that Neu5Ac serves as the minimal binding motif for VP1, which binds sialic acid residues in the context of α2,3 as well as α2,6 linkages. Baier et al. have recently reported co-crystal structures of polyomavirus VP1 protein complexed with divalent sialylated glycoamigomers on an oligoacrylamide scaffold, showing how these carbohydrates bind to the protein. However, conventional virus–receptor interaction studies of the recognition and binding process are generally limited to the intermolecular interaction between viral binding proteins such as VP1 and the glycan receptor.

Here, we have focused on the cross-linked aggregates formed by a well-defined carbohydrate and a well-characterized virus-like particle. We previously reported that bivalent and tetravalent glycodies serve as high-affinity cross-linkers of plant lectins. Multivalency greatly enhances the binding
affinity of a ligand even when the valency is relatively small, such as bivalent to tetravalent. In this study, we investigated the role of tetravalent sialo-glycoclusters as high-affinity cross-linkers of virus-like particles of MCPyV (MCPyV-LP). DLS and transmission electron microscopy (TEM) measurements were performed to evaluate the role of carbohydrate binding and to infer the possible clustering mode. Furthermore, we also report a novel simple membrane filtration assay for verification of the aggregates formed by tetravalent sialo-glycoclusters and polyomavirus.

### RESULTS

**Preparation of Ligands and Virus-like/Virus Particles.** We prepared a series of univalent, bivalent, and tetravalent glycosides as ligands (Figure 1 and Supporting Information, Figures S1–S6). Univalent and bivalent sialo-glycosides (1 and 2) bearing a Neu5Acα2,6Galβ1,4GlcNAc unit were synthesized enzymatically by adding Neu5Ac residues to 5-trifluoroacetamidopentyl-β-N-acetyllactosaminide (5-TFAP-β-LacNAc) and bis-5-[5′-(3″-thiobutanecarboxamido)pentanebcarboxamido]pentyl-β-LacNAc, respectively. Tetravalent asialo- and sialo-glycosides (3, 4, 5, and 6) were prepared by our previously described method (see the Supporting Information).36

MCPyV-LP, which forms uniform particles of 45 nm in diameter, was prepared by our previously described method.38 The JCPyV sample was prepared from cultured cells that were constitutively infected with the virus.39 The cell supernatant obtained after repeated freeze–thaw cycles was used in the filtration assay.

**DLS Measurements.** A solution of MCPyV-LP (0.01 mg/mL) was added to the DLS cell, and then, different concentrations of ligand solution (final concentrations: 0.031, 0.063, 0.25, 1.0, and 2.0 mM) were added. After each addition of ligand, the sample was reacted for 30 min at 25 °C. The DLS measurements for each sialylated ligand with MCPyV-LP are shown in Figure 2 and the Supporting Information, Figure S7.

In the absence of ligand, the average diameter of MCPyV-LP measured by DLS was 45 nm (Figure 2). The data showed that univalent 1 and bivalent 2 did not interact with MCPyV-LP (Supporting Information, Figure S7A,B). In the interaction of 5 with MCPyV-LP, the particle size distribution increased with increasing ligand concentration (Figure 2A). At 0.031 mM ligand concentration, two peaks were observed: one centered at ∼900 nm and the other at 45 nm, corresponding to the original peak of MCPyV-LP. On increasing the ligand concentration from 0.063 to 0.25 mM, the particle sizes increased (centered at 1100 and 1520 nm), and the original peak of MCPyV-LP disappeared. Complex formation reached a maximum at 0.25 mM ligand; thereafter, the size distribution decreased at 1.0 mM to give two broad peaks after 30 min with the main peak centered at 800 nm and a minor one at 70 nm.

Based on these results, the time course of the size distribution of the complex formed at the 0.25 mM ligand was further examined in detail (Figure 3 and Supporting Information, Figure S8). Plotting the size distributions formed by the 5–MCPyV-LP complex as a function of time showed that a small aggregate started to form after only 2 s of incubation with the particle size centered at 231 nm. Subsequently, the size distribution versus time plot showed a sigmoidal curve. Thus, the aggregate increased gradually at first with the particle size centered at 474 after 38 s and 580 nm after 2 min and then linearly over a period of ∼7 min. This
larger aggregate then slowly increased until a maximum particle size of 1520 nm was reached after 30 min. On the other hand, the behavior of the size distribution of complexes formed with 6 was considerably different from that of 5 (Figure 2B). There was no increase in the particle size distribution up to a ligand concentration of 0.25 mM. At the 1.0 mM ligand, two peaks were seen with the main one centered at 1924 nm and the minor at 45 nm, corresponding to the original peak of MCPyV-LP. At the 2.0 mM ligand, the main peak was ultimately focused at 1924 nm after the disappearance of the original peak. For the control tetravalent asialo-ligands 3 and 4, there was no increase in the MCPyV-LP particle size as expected (Supporting Information, Figure S7C,D).

**TEM Measurements.** Direct evidence of the formation of the 5–MCPyV-LP complex was obtained from negative staining TEM images (Figure 4). For TEM, we prepared the sample that showed the most efficient complex formation (final concentrations: 0.01 mg/mL MCPyV-LP and 0.25 mM 5). The resulting complex was observed as a dense aggregate of matter (~1500 nm wide) packed with virus-like particles (Figure 4A). At higher magnification, TEM revealed that the particles form in a cluster that is closely packed with the particles overlapping each other (Figure 4B). By contrast, for the solution of MCPyV-LP and the control tetravalent asialo-glycoside 3 ligand, TEM clearly showed isolated particles,
corresponding to those of MCPyV-LP in the absence of ligand (Figure 4C,D and Supporting Information, Figure S9). Thus, it seems likely that cross-linking MCPyV-LP via tetravalent ligands results in aggregated forms of virus particles. From these results, we assumed that the aggregates formed by the ligand–virus interaction involve cross-linking of MCPyV-LP via the tetravalent ligands, leading to clusters of virus particles. However, we needed to further verify whether or not the aggregates were produced by the specific binding of ligand 5 to the virus-like particles because the TEM images were obtained after the complex aggregates had been dried thoroughly.

**Membrane Filtration Assays.** The resulting aggregates were therefore subjected to a membrane filtration assay coupled with SDS-PAGE. Compound 5 (final concentration: 0.25 mM) was mixed with MCPyV-LP (final concentration: 0.01 mg/mL) and incubated for 30 min at 25 °C. The tetravalent glycocluster–virus mixture was then passed through a membrane filter to which MCPyV-LP is normally permeable. The resulting aggregates trapped on the filter membrane were then applied to SDS-PAGE using a silver staining method for visualization (Figure 5A). VP1 protein, the main component of polyomavirus, was readily detected in both the filtrate of MCPyV-LP solution and the solution before filtration (lanes 1 and 2 in Figure 5A). In contrast, the VP1 protein band was not observed in the filtrate of 5–MCPyV-LP complex solution (lane 3 of Figure 5A). Notably, the VP1 protein band was detected in the solution resulting from boiling the used filter membrane in 10 mM PBS (pH 7.4) containing 2-mercaptoethanol (5% w/v) for 10 min (lane 4 of Figure 5A). Furthermore, when ligand 6 (0.25 mM) and MCPyV-LP (0.01 mg/mL) were mixed, as shown in panel (d) of Figure 2B and applied to the membrane filtration assay, a band of VP1 protein was observed in the filtrate (Figure 5B). Collectively, these results indicate that the ligand 5–MCPyV-LP aggregates are efficiently trapped on a 0.2 μm sterilization membrane filter that is generally used for removal of bacteria.
Next, we evaluated the potential for trapping infectious JCPyV particles by mixing JCPyV derived from infected cells with the tetravalent glycocluster followed by membrane filtration. Compound 5 (final concentration: 0.25 mM) and JCPyV (10 HA infectious unit) were mixed, the interaction was allowed to proceed for 15 min, and the mixture was then passed through a sterilization filter. Viral DNA in the filtrate with or without premixing with compound 5 was determined by quantitative polymerase chain reaction (qPCR). In a comparison between JCPyV and JCPyV mixed with compound 5, the viral DNA level was markedly lower in the filtrate after the addition of ligand 5 to JCPyV (Table 1).

Table 1. Elimination of JCPyV by a Membrane Filtration Assay

| sample                     | DNA copy number   | elimination ratio (%) |
|----------------------------|-------------------|-----------------------|
| JCPyV                      | $2.38 \times 10^{4} \pm 108,292$ |                       |
| filtrate of JCPyV          | $2.19 \times 10^{6} \pm 114,250$ | 8                     |
| filtrate of the complex of JCPyV and 5 | $2.62 \times 10^{7} \pm 46,132$ | 89                    |

**DISCUSSION**

A series of univalent, bivalent, and tetravalent sialo- and asialo-glycosides (1–6) were prepared as potential ligands for MCPyV-LP using our previously reported method for the synthesis of tetravalent asialo-glycosides (3 and 4) and the corresponding sialo-glycosides (5 and 6) bearing short or long spacers between the glycan units and EGTA scaffold. In particular, the sialo-glycoclusters 5 and 6 are known to serve as high-affinity cross-linking ligands for *Sambucus sieboldiana* agglutinin (SSA), triggering the formation of large aggregates. This lectin is a heterotetrameric molecule with a hydrodynamic diameter of 12.2 nm and two sialic acid-binding sites at its extremities. In the present study, this ligand–lectin interaction was considered appropriate for the present ligand–virus from the viewpoint of the size of both acceptors. The hydrodynamic diameter of MCPyV-LP is 3.7 times larger than that of SSA. The 6′-SialylGal unit was chosen as the ligand because it was expected to act as a functional receptor for polyomaviruses. The nature of the ligand/virus-like particle complexes formed with these ligands was assessed by using DLS and TEM measurements. For the complexes formed by 5 or 6 bearing the sialic portion and MCPyV-LP, the particle sizes increased with increasing ligand concentrations until a maximum size centered at 1500–1900 nm was reached. In the case of excess ligand 5, a decrease in complex size was observed after the maximum was reached (see panel (e) of Figure 2A). This indicates that the agglutination process can be reversed by adding an excess amount of ligand to act as an antagonist because these profiles were comparable to those produced by ligand–lectin (SSA or ECA) complexes from our previous data. Taking into account the TEM images, we conclude that the present ligands 5 and 6 work as cross-linkers of the virus-like particles, resulting in the formation of large aggregates. In addition, the complex formation of 5 bearing a short spacer was shown to be much faster than that of 6 with a long spacer, indicating that the cross-linking is sensitive to the flexibility of the spacer to the sugar epitopes. From these results, it is considered that this kind of ligand–spacer combination is favorable for binding site occupation based on the previously presented divalent sialylated glycoconjugates binding to polyomavirus VP1 protein using X-ray crystallography.

We further examined on how the complex between ligand 5 and MCPyV-LP grew into a large aggregate (Figure 3 and Supporting Information, Figure S8). Based on previous information on the agglutination mechanism underlying ligand–lectin interactions, an outline of the agglutination of MCPyV-LP by ligand 5 is shown in Scheme 1. The binding process proceeds in two stages during agglutination. The first step initiates quickly, leading to the formation of a small aggregate in which some virus particles become cross-linked to each other through ligand 5. The average diameter of the resulting small aggregates (231 nm) corresponds to about 5 times the diameter of MCPyV-LP. The formation of such small aggregates, which act as a trigger in sequence, is the rate-limiting step in the overall process of agglutination. In the second step, the resulting small aggregates bind cooperatively to similar other aggregates via ligand 5, thereby growing into larger particles arranged in a network of small aggregates because the size distribution curve is sigmoidal rather than hyperbolic (Figure 3). As a result, the engagement of multiple binding sites of the VP1 capsid on the surface of MCPyV-LP will lead to an enhancement of the cross-linking imparted by the favorable orientation of the target ligand (Scheme 1). To our knowledge, this is the first report to elucidate the growth
process of agglutination for ligand–virus complexes characterized by DLS.

Our DLS and TEM results imply that the tetravalent glycoclusters are capable of transforming nanoscale virus particles in solution into microscale aggregates. In general, virus particles are nanoscale fine particles; therefore, a special device such as an ultracentrifuge is required for separation and purification. By contrast, microscale molecules (e.g., microorganisms) can be separated by using a general sterilizing filter. In the present study, we demonstrated that the microscale cross-linked ligand–virus complexes were trapped by an ordinary sterilizing filter, whereas MCPyV-LP alone was not trapped (Figure 5). Further, the technique developed in this study enabled us to trap not only virus-like particles but also infectious particles of JCPyV efficiently via membrane filtration (Table 1). In other words, this approach can be used as a purification technique for pathogenic viruses. Several methodologies have been developed for purification of virus particles by using density gradient ultracentrifugation, precipitation method, and chromatography methods. The present membrane filtration assay might also be applied to develop a high-performance instrument for blood purification, which would be useful to remove several viruses with binding affinity for sialylated glycans, thereby contributing to the prevention of viral diseases such as progressive multifocal leukoencephalopathy, which is occasionally observed in patients with acquired immune deficiency syndrome and in those with multiple sclerosis treated with monoclonal antibody therapies.

■ CONCLUSIONS

We have demonstrated the agglutination of nanoscale human polyomavirus by using a low-molecular tetravalent sialo-glycocluster as a cross-linker. These results are valuable and important because they enhance our knowledge of effective ligand–virus interactions that can be used as a purification technique for a variety of viruses.

■ EXPERIMENTAL PROCEDURES

Ligands. The monovalent sialo-ligand (1) and the bivalent sialo-ligand (2) were synthesized by enzymatically α2,6-sialylating the previously reported compound 5-TFAP-β-LacNAc44 and bis-5-{5′-[3′-thiobutanecarboxamido]-pentanecarboxamido}pentyl-β-LacNAc,45 respectively (see the Supporting Information).

DLS Measurements. MCPyV-LP (0.02 mg/mL) in 10 mM PBS (pH 7.4) was filtered (0.20 μm), and the concentration was determined by absorbance of ultraviolet light at 280 nm. Ligand solutions were prepared at concentrations between ~0.063 and 4.0 mM in 10 mM PBS. Next, 35 μL of each ligand solution and polyomavirus-like particle solution were mixed. After incubation for 30 min at 25 °C, the particle sizes of the solutes were measured by DLS at the same temperature by using a Zetasizer Nano ZSP instrument (Malvern Instruments, Worcestershire, UK).

TEM Measurements. MCPyV-LP (0.02 mg/mL) in 10 mM PBS (pH 7.4) was filtered (0.20 μm). Tetravalent asialo- (3) and sialo- (5) ligands (0.5 mM) were dissolved in the same buffer and mixed with an equal volume of MCPyV-LP solution. After incubation for 30 min at 25 °C, the ligand–MCPyV-LP mixture was deposited onto a formvar-coated copper grid and negatively stained with 2% uranyl acetate solution. Samples were observed by using a JEM-1400Plus electron microscope (JOEL) operating at an acceleration voltage of 100 kV. Digital images (3296 × 2472 pixels) were captured by using a charge-coupled device (CCD) camera.

Membrane Filtration Assays. First, a mixture containing 0.01 mg/mL MCPyV-LP and 0.25 mM compound 5 or 6 in 10 mM PBS (pH 7.4) was incubated for 30 min at 25 °C and then passed through a membrane filter with a 0.2 μm pore size. Four samples corresponding to filtered MCPyV-LP, compound 5–MCPyV-LP aggregate solution and filtrate, and the filter membrane wash fraction were analyzed by SDS-PAGE. The filter membrane wash fraction was prepared by boiling the filter membrane in 10 mM PBS (pH 7.4) containing 2-mercaptoethanol (5% w/v) for 10 min. SDS-PAGE was carried out on a 13% polyacrylamide gel, according to the method of Laemmli.46 The gels were stained by the silver staining method to visualize viral proteins. The molecular mass of the VP1 protein was estimated by including size standards (10–250 kDa; Precision Plus Protein Kaleidoscope Standard; Bio-Rad Laboratories, Inc., Carlsbad, CA) on the gels.

Second, the tetravalent sialo-ligand (10 μL, 0.5 mM in PBS) was added to JCPyV (10 μL, 51 HA infectious unit/50 μL) in a microtube. After incubation for 15 min at room temperature, the mixture was suspended in 45 μL of 10 mM PBS (pH 7.4). The suspension was then filtered through a 0.2 μm membrane filter, and the final volume of the filtrate was adjusted to 400 μL with 10 mM PBS (pH 7.4). The amount of JCPyV DNA was quantified by qPCR, as previously described, using an ABI PRISM 7900HT instrument (Applied Biosystems, Foster City, CA, USA). For quantification of JCPyV DNA, the M1-IMRb plasmid DNA was serially diluted to 10−6–10−8 copies per reaction to generate a linear standard curve.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c03269.

Analytical methods, ligands, 1H NMR spectrum/integral value and 13C NMR spectrum of ligands, DLS measurements, and TEM images (PDF)

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Notes

The authors declare no competing financial interest.

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