Design of a Sialylglycopolymer with a Chitosan Backbone Having Efficient Inhibitory Activity against Influenza Virus Infection

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We verified here the inhibitory activity of a sialylglycopolymer prepared from natural products, chitosan and hen egg yolk, against influenza virus infection and estimated the requirements of the molecule for efficient inhibition. The inhibitory activity clearly depended on two factors, the length (the degree of polymerization: DP) of the chitosan backbone and the amount (the degree of substitution: DS) of conjugated sialyloligosaccharide side chain. The inhibitory efficiency increased in accordance with the DP value, with the highest inhibitory activity obtained when the DP was 1430. The inhibition of virus infection reached more than 90% as the DS value increased up to 15.6% when the neighboring sialyloligosaccharide side chains came as close as 4 nm, which was nearly the distance between two receptor-binding pockets in a hemagglutinin trimer. These results demonstrate that the sialylglycopolymer could be an excellent candidate of the safe and efficient anti-influenza drug.

Introduction

Influenza viruses express two envelope proteins, hemagglutinin (HA)[a] and neuraminidase (NA). HA plays a key role in cell recognition and initiating infection by binding sialic acid-containing receptors on host cells, which then mediates the subsequent membrane fusion and viral entry. It recognizes specific sugar chain structures including sialic acids at the nonreducing termini of *N*- and *O*-glycans, such as Neu5Acα2−3(6)Galβ1−3(4)GlcNAcβ1−.[1,2] NA is responsible for the release of progeny viruses by the removal of sialic acid from the surface of virions as well as infected cells, preventing aggregation of the progeny virus particles.

Influenza virus is one of the most important human pathogens, as it not only causes high morbidity and mortality particularly in the elderly population, but it also imposes a considerable economic burden worldwide. Present medicinal methods for prevention and therapy include vaccination and anti-influenza medication, however, neither are entirely satisfactory.[1] The current vaccines do not provide a complete solution because of their limited efficacy in preventing infection and the frequent emergence of genetic variant viruses every year. One of the most promising alternatives to inhibit virus infection is use of antiviral drugs that block the activities of HA and NA. The recently developed specific inhibitors for influenza NA, zanamivir and oseltamivir, are now widely utilized but provide a limited beneficial effect because they can only prevent the function of NA after the influenza virus infects host cells. Moreover, there is the possibility that resistant viruses against the drugs will appear, and in fact, resistance in up to 2% of patients has already been reported in clinical trials of oseltamivir.[3] Moreover, these NA inhibitors are also under investigation as a suspected cause of neuroexcitatory actions leading to death.[4] As well, the NA inhibitors are essentially effective only after the onset of infection in the host and therefore are not suitable for prophylactic purposes. Although many approaches have been taken to prevent infection by blocking the HA-binding to the host cell receptors using monovalent sialoside analogues like NA inhibitors, success has not been achieved, probably due to the conformation of the binding pocket of HA which is shallower and less charged than that of NA.[5] Since 1997, when the H5N1 avian influenza virus first invaded human society, the reported cases of human infection by highly pathogenic avian influenza viruses are increasing. It is extremely important to take a high level of precautionary measures should a pandemic of a new influenza virus with high pathogenicity and new antigenicity occur. Thus, development of anti-influenza drugs applicable to preventive purposes as well as remedial treatment is urgently necessary.

We recently reported the synthesis of sialylglycopolymer with a chitosan backbone, CDO-chitosan (complex disialooligosaccharide−chitosan).[6] The agent was enzymatically synthesized from *N*-linked sialylglycopeptide (SGP) from hen egg yolk possessing two Neu5Acα2−6Galβ1−4GlcNAcβ1− moieties at its nonreducing termini.[7] This was achieved using the transglycosylation activity of the recombinant endo-β-*N*-acetylglucosaminidase from *Mucor hiemalis* (Endo-M),[8–10] followed by chemical conjugation to a chitosan like multivalent pendant. Compared with a monomeric sialoside like

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[a] Abbreviations: AGTG, asialyloligosaccharide transglycosylation product toward *p*-FP-GlcNAc; A/New Caledonia, A/New Caledonia/20/99; A/Panama, A/Panama/2007/99; B/Shanghai, B/Shanghai/361/2002; CDO-chitosan, complex disialooligosaccharide−chitosan; CIU, cell-infecting unit; DMEM, Dulbecco's modified Eagle medium; DP, degree of polymerization; DS, degree of substitution; Endo-M, endo-β-*N*-acetylglucosaminidase from *Mucor hiemalis*; HA, hemagglutinin; MDCK, Madine−Darby canine kidney; NA, neuraminidase; *p*-FP-GlcNAc, *p*-formylphenyl 2-acetamido-2-deoxy-β-D-glucopyranoside; SGP, sialylglycopeptide; SGTG, sialyloligosaccharide transglycosylation product toward *p*-FP-GlcNAc; 6′-SLN, 6′-sialyllactosamine.

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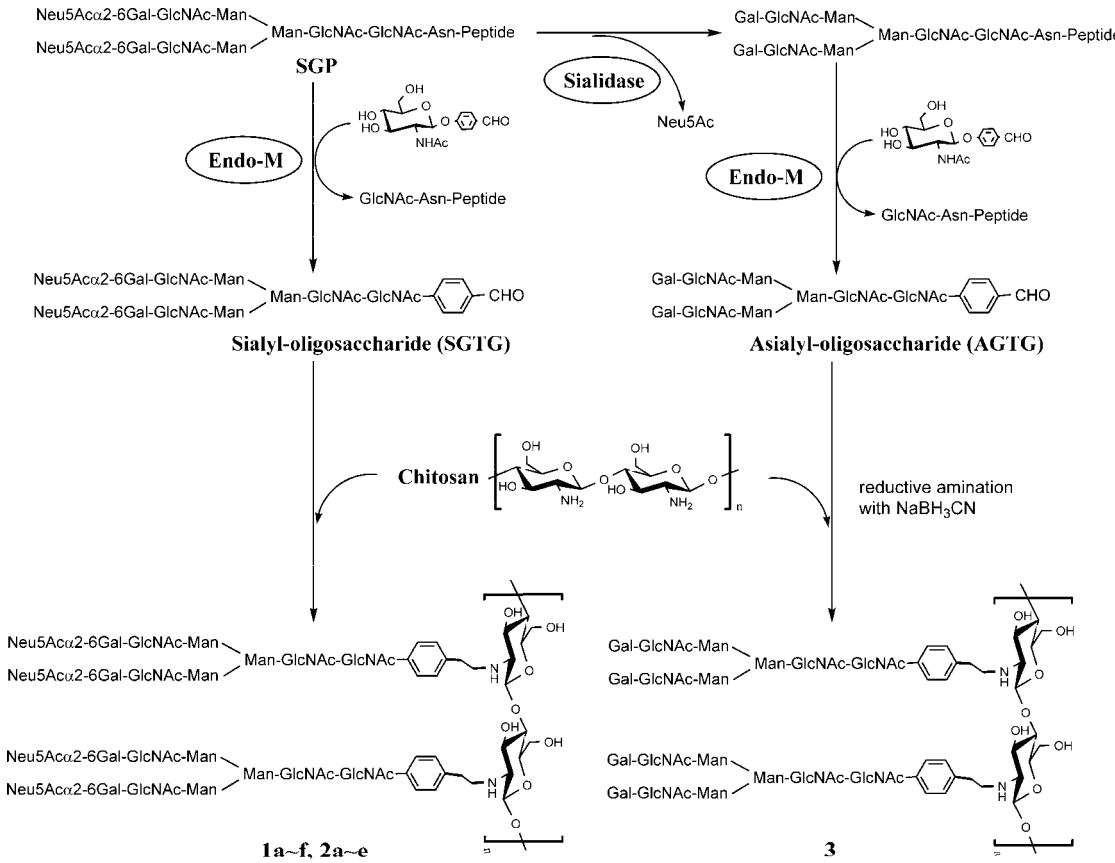

Figure 1. Synthesis of sialyl- and asialyl-glycopolymer with chitosan backbones.

sialyllactose which binds HA with a relatively high dissociation constant of 2 mM, polyvalent sialic acid compounds obtained by conjugation to polyacrylamide,[11,12] poly(acrylic acid),[13] polystyrene,[14] poly(α-L-glutamic acid),[15] and γ-polyglutamic acid,[16] have demonstrated enhanced binding affinity against HA by the so-called glycoside cluster effects.[17] Multiple interaction of multivalent sialosides on a single molecule with receptor binding sites of HA trimers on a virus particle shows much stronger avidity than the sum of each binding owing to this effect.[18,19] We should be circumspect, however, in using the synthetic polymers such as polyacrylamide, poly(acrylic acid), as well as polystyrene in humans due to their known cytotoxicity[20] and immunogenecity.[21] On the other hand, chitosan is a polysaccharide obtained from *N*-deacetylated derivatives of chitin, a compound that is widely distributed in nature and used in food products for health improvement. In the previous report, we confirmed that CDO-chitosan possessed high affinity to HA and hemagglutination inhibition activity.[6]

In this study, with the aim to develop a practically useful compound to inhibit influenza virus infection, we investigated the multivalent property of the CDO-chitosan molecule required for effective inhibition. These included the length of the chitosan backbone and the amount of the sialyloligosaccharide chains conjugated to it by examining the suppression of infectivity of type A and B influenza viruses in cell culture. This study helped us to estimate the characteristics of CDO-chitosan essential for effective blockage of the binding of influenza virus HA to cellular receptors. Finally, the most appropriate CDO-chitosan molecule as an influenza virus inhibitor was discussed from a mesoscopic point of view using its computer-simulated molecular model.

Table 1. Synthesized Glycopolymers with Various Lengths of the Chitosan Backbone (DP) and the Degree of Substitution of the Sialyloligosaccharide (DS)

type	sugar moiety	DP of chitosan[a]	DS (%)[b]
1a	Neu5Acα2−6Galβ1−4GlcNAcβ1−	393	−[c]
1b	Neu5Acα2−6Galβ1−4GlcNAcβ1−	5990	10.3
1c	Neu5Acα2−6Galβ1−4GlcNAcβ1−	1430	16.9
1d, 2b	Neu5Acα2−6Galβ1−4GlcNAcβ1−	401	15.6
1e	Neu5Acα2−6Galβ1−4GlcNAcβ1−	116	12.3
1f	Neu5Acα2−6Galβ1−4GlcNAcβ1−	20	57.0
2a	Neu5Acα2−6Galβ1−4GlcNAcβ1−	401	35.8
2c	Neu5Acα2−6Galβ1−4GlcNAcβ1−	401	8.4
2d	Neu5Acα2−6Galβ1−4GlcNAcβ1−	401	3.3
2e	Neu5Acα2−6Galβ1−4GlcNAcβ1−	401	2.1
3	Galβ1−4GlcNAcβ1−	393	−[c]

[a] The degree of polymerization of chitosan was estimated from its MW. [b] Estimated by ^{1}H NMR spectroscopy. [c] Not estimated.

Results

Inhibitory Activity of CDO-Chitosan against Influenza Virus Infection. CDO-chitosan is a sialylglycopolymer composed of a chitosan backbone and multivalent sialyloligosaccharide side chains (Figure 1). At first, we synthesized CDO-chitosan (**1a**, Table 1) and asialyl-CDO-chitosan (**3**, Table 1) using chitosan with an average degree of polymerization (DP) (where DP is defined as the polymerized number of β1,4-linked D-glucosamine residues per molecule) of 393. The syntheses of both compounds were carried out under the same reaction conditions in which a molar ratio of synthesized sialyloligosaccharide transglycosylation product toward *p*-formylphenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (*p*-FP-GlcNAc) (SGTG) or asialyloligosaccharide transglycosylation product toward

p-FP-GlcNAc (AGTG) was both 40% of D-glucosamine residues of the chitosan backbone. It was assumed that the degree of substitution (DS) (where DS is defined as the ratio of D-glucosamine residues conjugated with sialyloligosaccharide chains over the total number of D-glucosamine residues in the chitosan) was almost the same between **1a** and **3**.

We estimated the inhibitory activity of CDO-chitosan by a virus inhibition assay using Madine−Darby canine kidney (MDCK) cells. Each inhibitor solution was mixed with an influenza virus solution of A/New Caledonia/20/99 (A/New Caledonia), A/Panama/2007/99 (A/Panama), or B/Shanghai/361/2002 (B/Shanghai) for 1 h at 25 °C, and then applied to MDCK cells on a 96-well plate. The infected cells were stained by an indirect immunofluorescent method using anti-influenza virus antibodies and counted to estimate the infectivity (Figure 2). The inhibitory activity of the CDO-chitosan against the three viral strains were dose-dependent and much higher than those of fetuin and 6′-sialyllactosamine (6′-SLN). The IC_{50} values of CDO-chitosan against A/New Caledonia, A/Panama, and B/Shanghai were 0.02, 0.4, and 0.1 mg/mL, which were quite low compared with those of fetuin, which were 9, 1, and 10 mg/mL, respectively (Table 2.) CDO-chitosan almost completely inhibited the infection of A/New Caledonia at a concentration of 0.25 mg/mL, and the residual infectivity against A/Panama and B/Shanghai at a concentration of 1 mg/mL was about 20% and 8%, respectively, indicating that CDO-chitosan was most effective against A/New Caledonia. In contrast, asialyl-CDO-chitosan had no inhibition activity against both A/New Caledonia and A/Panama and exhibited only limited inhibition against B/Shanghai at 1 mg/mL, the highest concentration tested. The results clearly demonstrate that CDO-chitosan inhibits influenza virus infection via its sialic acid moiety through binding to HA.

Monomeric 6′-SLN had IC_{50} values of 2, 3, and 10 mg/mL against A/New Caledonia, A/Panama, and B/Shanghai, which were 100, 7.5, and 100 times higher than that of CDO-chitosan, respectively (Table 2). These values were much higher than those of CDO-chitosan. Because the molecular weights of the chitosan (63600) and SGTG (2327) are much greater than that of 6′-SLN (675), the activity per molecule of the sialic acid contained within the CDO-chitosan should be much higher compared with that of 6′-SLN (Table 2, see the next section). This observation led to the conclusion that the multivalency of the sialic acids caused an increase in inhibition.

Monomeric 6′-SLN inhibited the infection of A/Panama almost equally to that of A/New Caledonia. CDO-chitosan holding multivalent sialic acids, however, inhibited A/Panama less effectively than A/New Caledonia, suggesting that the effect of multiple sialic acids is not simple and varies depending on the influenza viral strains, namely on the structure of HA especially around the receptor binding pocket. Therefore, we then studied the properties of a CDO-chitosan molecule required for effective suppression of influenza virus infectivity.

Dependence of Inhibitory Activity on the Length of the Chitosan Backbone. To examine how the inhibitory activity of CDO-chitosan is affected by the length of the chitosan backbone, we synthesized inhibitors with 5 kinds of chitosan, each with different average DP values (5990, 1430, 401, 116, and 20). The DS values were estimated by NMR spectroscopy as 10.3%, 16.9%, 15.6%, 12.3%, and 57.0% for CDO-chitosan with DP values of 5990, 1430, 401, 116, and 20, respectively (**1b**−**f**, Table 1). Although the DS value differs in these samples, the inhibitory activity will not be affected so much by that except

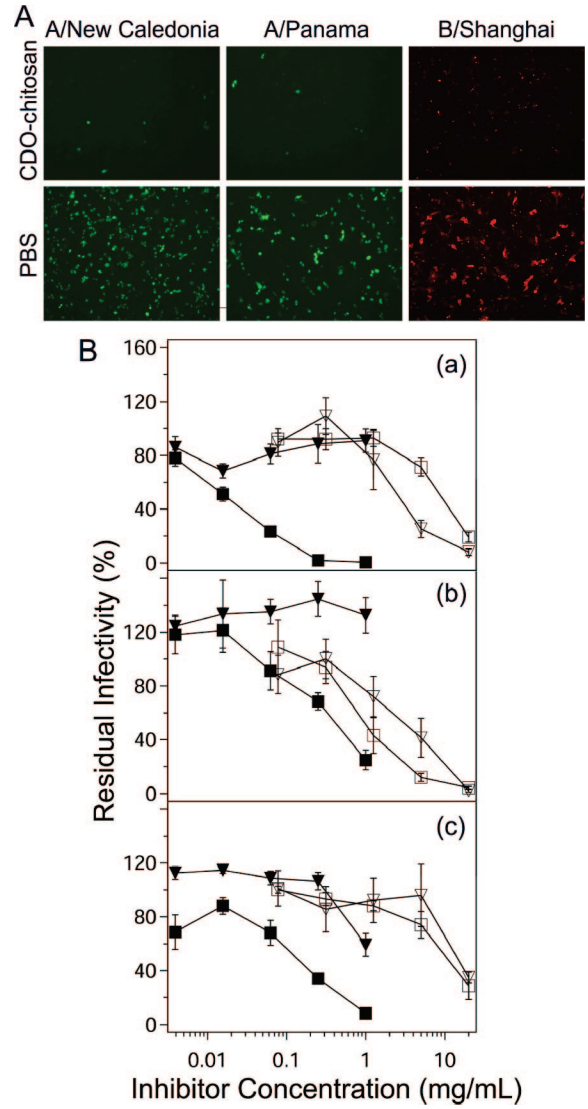

Figure 2. Inhibitory activity of CDO-chitosan against influenza virus infection of MDCK cells. (A) Detection of MDCK cells infected with influenza viruses. A/New Caledonia/20/99 (H1N1) (left), A/Panama/2007/99 (H3N2) (middle), or B/Shanghai/361/2002 (right) was inoculated into MDCK cells after incubation with CDO-chitosan (**1a** in Table 1) at a concentration of 1 mg/mL (top panels) or PBS (bottom panels). Infected cells were stained by the indirect immunofluorescent method as described in Materials and Methods. (B) Effect of multiplication of the sialic acid on the inhibitory activity against influenza virus infection. Serially 4-fold diluted CDO-chitosan (**1a**, ■), asialyl-CDO-chitosan (3, ▼), 6′-sialyllactosamine (▽), or fetuin (□) were mixed with A/New Caledonia (a), A/Panama (b), or B/Shanghai (c) and then inoculated into MDCK cells. The residual infectivity was displayed as the ratio (%) compared to that where the viruses were incubated with PBS (means ± SD, $N = 4$).

the longest and the shortest samples (see the next section). Using these CDO-chitosans, the virus inhibition assay was performed.

The CDO-chitosans showed DP dependence in their inhibitory activities (Figure 3). We found that the inhibitory effect against all three strains became more efficient as the DP increased (the length of chitosan backbone became longer) from 20 to 1430, reaching almost 100% inhibition at a concentration of 1.0 mg/mL of CDO-chitosan with a DP of 1430. The IC_{50} values of the CDO-chitosans against A/New Caledonia, the most sensitive strain to the multivalency of sialic acid, decreased in a logarithmic fashion with the length as follows: 0.2 mg/mL (DP 20), 0.04 mg/mL (DP 116), 0.01 mg/mL (DP 401), and 0.005

Table 2. IC_{50} Values of CDO-Chitosan in Inhibitory Assay of Influenza Virus Infection of MDCK Cells

inhibitors		IC_{50} (mg/mL)			IC_{50} (sialic acid μM)[a]		
type	DP[b]	A/New Caledonia /20/99	A/Panama/2007/99	B/Shanghai /361/202	A/New Caledonia/20/99	A/Panama /2007/99	B/Shanghai /361/202
1a	393	0.02	0.4	0.1	*c*	*c*	*c*
1b	5990	0.02	0.3	0.2	10	155	103
1c	1430	0.005	0.1	0.03	3	61	18
1d	401	0.01	0.3	0.09	6	179	54
1e	116	0.04	>1	0.4	22	>550	221
1f	20	0.2	0.6	*d*	154	463	*d*
6′-SLN		2	3	10	3000	4000	14800
fetuin		9	1	10	720[e]	80[e]	800[e]

[a] Concentration of sialic acid in assay mixture. [b] DP of chitosan backbones. [c] Not estimated due to the unknown DS value of **1a**. [d] Not determined due to no inhibitory activity observed. [e] Estimated from the amount of α2,6 type sialylgalactose in *N*-linked oligosaccharide bound to fetuin.[38]

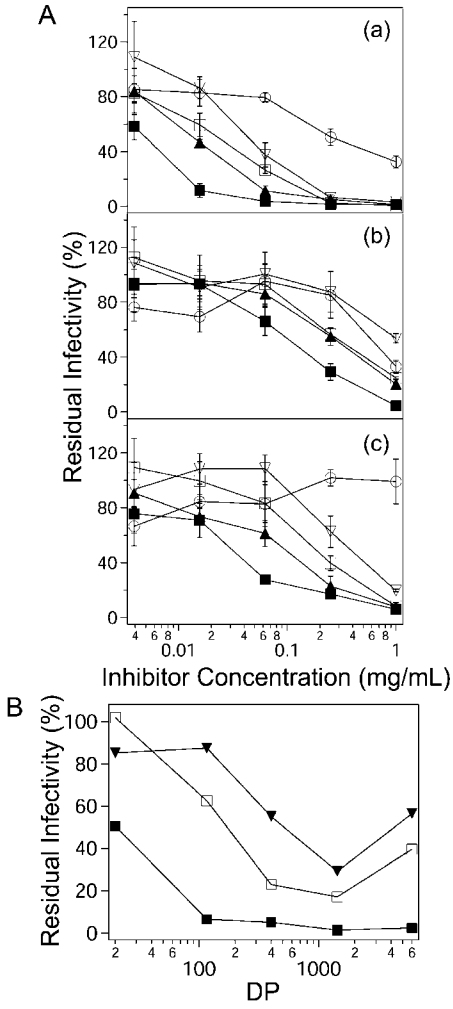

Figure 3. Effect of the length of the chitosan backbone on the inhibitory activity of CDO-chitosan against influenza virus infection of MDCK cells. A. Inhibition of influenza virus infection by CDO-chitosan prepared with various lengths of chitosan backbone. Serially 4-fold diluted CDO-chitosan preparations whose DP values were 5990 (**1b**, □), 1430 (**1c**, ■), 401 (**1d**, ▲), 116 (**1e**, ▽), and 20 (**1f**, ○), respectively, were incubated with A/New Caledonia (a), A/Panama (b), or B/Shanghai (c) and inoculated into MDCK cells. The residual infectivity was indicated as shown in Figure 2 (means ± SD, $N = 4$). (B) Dependence of inhibitory activity of the CDO-chitosan on the DP value. The residual activity of A/New Caledonia (■), A/Panama (▼), and B/Shanghai (□) treated with each CDO-chitosan at a concentration of 0.25 mg/mL in A was plotted against the DP values.

mg/mL (DP 1430), which were 10, 50, 200, and 400 times lower than that of 6′-SLN (Table 2). When IC_{50} values were evaluated as sialic acid concentration (IC_{50} (sialic acid μM)), the ratio of CDO-chitosan to 6′-SLN showed further lower values by two or three times than IC_{50} (mg/mL). IC_{50} (sialic acid μM) again decreased as the chitosan backbone became longer not only with A/New Caledonia but also with A/Panama and B/Shanghai, clearly indicating the enhancement of glycoside cluster effect as the DP value increased (Table 2). The CDO-chitosan with a DP of 5990 was, however, less or equally effective than the one with a DP of 1430 (Figure 3B). One of the explanations for this phenomenon could be that the CDO-chitosan with a DP of 5990 had a lower DS value of 10.3% compared with the one with a DP of 1430 having a DS value of 16.9% (see the next section). The low DS value of the longest CDO-chitosan is probably due to the hydrophobic characteristics of chitosan, that is, the longer chitosan becomes, the lower becomes the solubility in water. The quite low solubility of the longest chitosan might not permit the efficient addition of the sialyloligosaccharide moiety in the reductive amination reaction.

The multivalent effect of sialic acid on the inhibition of influenza virus infection differed among strains. Nevertheless, the dependency of CDO-chitosan on the DP value was similar in all three strains, namely the complex with a DP of 1430 blocked virus infection most efficiently. Considering that chitosan becomes less soluble as the DP increases, chitosan with a DP around 500 is still suitable for the synthesis of CDO-chitosan because it is easy to dissolve in water or mildly acidic water without a loss of significant inhibitory activity.

Dependence of the Inhibitory Activity on Amount of the Sialyloligosaccharide Conjugated to Chitosan Backbone. Next, to examine how the inhibitory activity depends on the amount of the sialyloligosaccharide chain conjugated to chitosan, we prepared CDO-chitosans by mixing SGTG with chitosan at molar ratios of 80, 40, 20, 10, and 5%. According to the result above, we used chitosan with a DP of 401. The DS values of each preparation estimated by NMR spectroscopy were 35.8%, 15.6%, 8.4%, 3.3%, and 2.1%, respectively (Table 1).

There was a clear relationship that the inhibitory activity increased as the DS value became greater, although the degree of dependency on DS differed among virus strains (Figure 4). Infection of A/Panama was most strikingly affected by the DS value. It was hardly inhibited by the CDO-chitosan with a DS of 2.1%, but the CDO-chitosan with a DS of 15.6% blocked its infection by more than 90%. On the other hand, the inhibition against infection of A/New Caledonia was independent of the DS in CDO-chitosan when the value was above 3.3%. However, there was no increase in the inhibition against all of the three viruses, when the chitosan backbone was conjugated with sialyloligosaccharide with a DS over 15.6% (see discussion).

Discussion

The inhibition of influenza virus infection by blocking receptor binding with analogues of the ligand sialic acid has not yet been successful, probably due to the high dissociation constant of the analogues against the relatively shallow binding

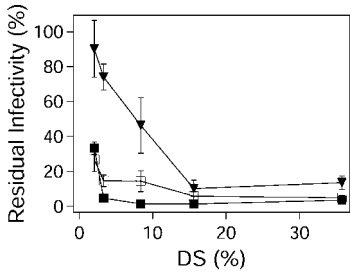

Figure 4. Dependence of the inhibitory activity of CDO-chitosan against influenza virus infection on the DS value. A/New Caledonia (■), A/Panama (▼), and B/Shanghai (□) were preincubated with CDO-chitosan (DP 401) carrying various amount of sialyloligosaccharide side chain (**2a**−**e** in Table 1) at a concentration of 1 mg/mL and then inoculated into MDCK cells. The DS values of each preparation were estimated from NMR spectroscopy. The residual infectivity was estimated as shown in Figure 2 (means ± SD, $N = 4$).

pocket of HA.[5] One way to solve the problem may be to conjugate multivalent sialic acids to a backbone polymer to achieve the glycoside cluster effect on binding to viruses, but such sialyloligosaccharide-containing polymers are still in development and are not ready for a practical application in humans. One of the reasons for this is that the safety of these polymers has not yet been proven. CDO-chitosan should be suitable for use in humans because its major components are derived from natural components. In this study, we verified the effectiveness of CDO-chitosan for inhibiting the infection of influenza viruses using cell culture.

The most effective length of the chitosan backbone was a DP of 1430. Longer chitosan molecules exhibited difficulty in the CDO-chitosan synthesis, probably because they do not dissolve in water and therefore resulted in an insufficient DS value through incomplete amination reaction for adding sialyloligosaccharide side chains. On the basis of the observation that chitosan with a DP of 1430 is already hard to dissolve in mildly acidic water, we selected chitosan with a DP around 500 as a suitable candidate for the synthesis of CDO-chitosan. CDO-chitosan with a DP of 401 and a DS of 15.6% demonstrated satisfactory inhibitory activity against all the three strains tested in this study.

We performed the molecular dynamics simulations for the sialyloligosaccharide moiety and a chitosan with a DP of 10 in explicit water molecules, followed by the conjunction of them (Figures 5A, 5B). The molecular model will help us to understand the interaction between CDO-chitosan and HA (Figure 5C,D). The picture of the simulated CDO-chitosan bound to a HA trimer of H1 subtype[23] demonstrates to us that the length of chitosan with a DP of 10 is close to that of a side of the HA trimer head. Therefore it is easy to speculate that the cluster effect hardly occurs with the CDO-chitosan with the DP of 20 because it can hardly reach the adjacent HA trimer. A mesoscopic point of view presents how CDO-chitosan works as a binding-inhibitor against influenza virus (Figure 6). As the length of a D-glucosamine unit in chitosan is ca. 0.6 nm,[22] the length of chitosan with a DP of 500 is 300 nm. Considering that the inhibitor with a DP of 116 (ca. 70 nm) did not exhibit sufficient inhibitory activity, the chitosan backbone required for inhibition should be longer than the diameter of the particle (about 100 nm). The most important feature of CDO-chitosan for the inhibition, thus, is considered to be the length of the fiber-like structure much longer than the diameter of the virus particle. The virus particles captured by CDO-chitosan should be prevented from approaching the cell surface by the "long tail" of the fibrous molecules.

The molecular structure of the CDO-chitosan reflecting the DS around 15% shows that the distance between two sialic acids in each neighboring SGTGs is ca. 3.5 or 4 nm (Figure 5A,B). HA is a trimeric protein having a sialic acid binding site in each subunit (Figure 5D),[24] and the distance between each site is also close to 4 nm.[23] Therefore, two SGTGs in the CDO-chitosan with DS of 15% can fit two of the three sialic acid binding sites in HA (Figure 5C,D). Because these two sites are the closest pair of receptor binding pockets on the surface of influenza virus particle, no more addition of SGTGs is required for the glycosidic cluster effect. It is therefore reasonable that the sialyloligosaccharide conjugated with chitosan above a DS of 15.6% did not yield greater inhibitory activity. Thus the CDO-chitosan with a DS of 15.6% yielded sufficient inhibition even against A/Panama strain that was the most resistant strain to the inhibitor.

When the CDO-chitosan complex is made of chitosan with a DP of 500 and a DS of 15%, its MW must be ca. 219600. The number of CDO-chitosan molecules in 1 mL at a concentration of 1 mg/mL is thus 2.4×10^{15}. One of these CDO-chitosan complexes thus occupies a cubic volume with sides of about 75 nm. Considering that the length of the chitosan backbone is 300 nm, we can image a condition where there is a high density of the inhibitor in a 1 mg/mL solution (Figure 6, below). Because the titer of the virus solution was 4.0×10^5 cell-infecting units (CIU)/mL, the number of CDO-chitosan complexes per virus particle is ca. 7.5×10^9. Considering that chitosan has both straight and rigid properties, influenza virus particles should easily come into contact with the arms of the sialic acid protruding from the chitosan backbone during swimming in the mesh of the inhibitor.

CDO-chitosan demonstrated limited inhibition against A/Panama (H3 subtype) compared with A/New Caledonia (H1 subtype). This result could not be due to the weak affinity between the sialic acid moiety and the HA molecule of A/Panama[2] because monomeric 6′-SLN showed higher inhibitory activity against A/Panama. Instead, it rather indicates that the multivalent effect of CDO-chitosan did not function well on A/Panama. It is notable that inhibition of A/Panama infection was strongly dependent on the DS value (Figure 4). Interaction of the sialyloligosaccharide side chain in CDO-chitosan to HA is affected not only by the intrinsic binding affinity between the two molecules but also the accessibility of the sialic acid to HA due to the fixation of the side chains on the chitosan backbone. At high DS value, however, such hindrance should be relieved. Chitosan backbone has a helical ribbon form of glycosidic rings with amino groups on each side alternately,[25] thus the sialyloligosaccharide moieties come to protrude in all directions to cover the space widely around the chitosan backbone as the DS value increases (Figure 5E). When the DS value is high, therefore, the side chains will greatly increase their chance to come into contact with HA molecules, resulting in the high inhibitory activity. Related to this, it should be considered that the inhibitory activity of CDO-chitosan is dependent on the length of the sialyloligosaccharide side chain.

In December 2003, a highly pathogenic avian influenza virus began to spread widely in poultry throughout countries in Asia, the Middle East, Europe, and Africa, resulting in huge economic losses for the poultry industries. Following this event, the opportunity of transmission of the virus to humans began to increase, and since 1997 when the first invasion by the highly pathogenic H5N1 influenza A virus occurred in Hong Kong, almost 350 human infections have been identified, of which more than 200 have been fatal, raising serious worldwide

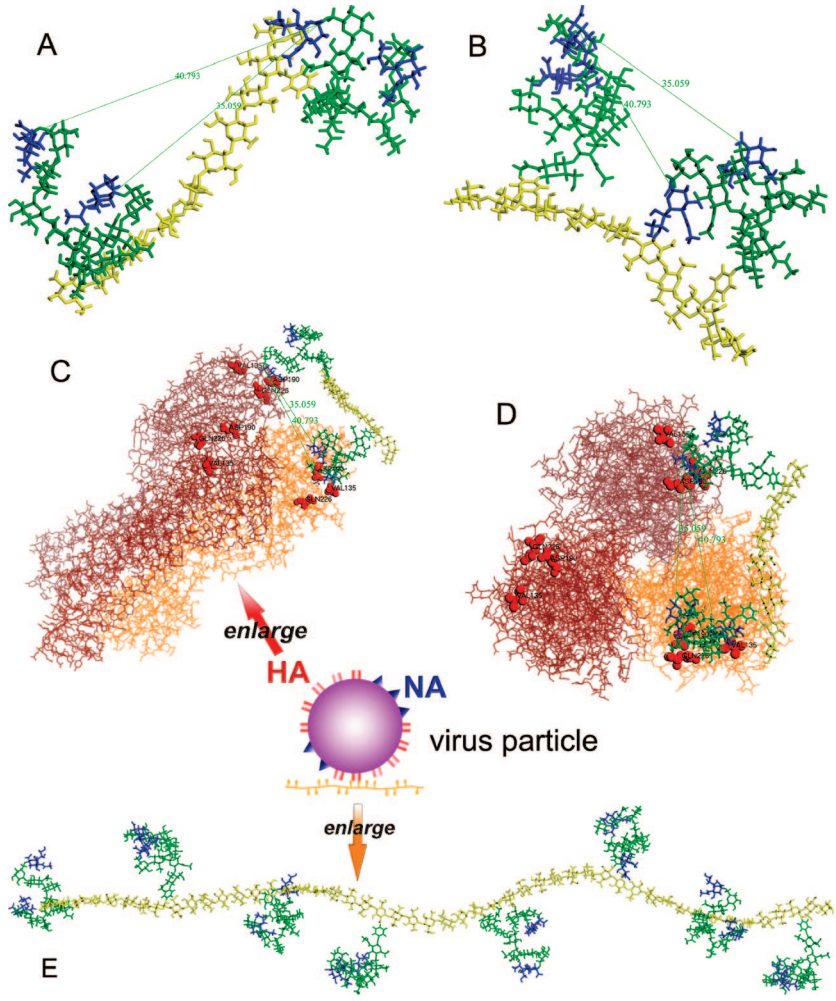

Figure 5. The structure of CDO-chitosan built by molecular modeling. Figures were constructed with models of SGTG and chitosan with a DP of 10, each of which was calculated by molecular dynamics simulation for 5 ns in explicit water molecules. The SGTG and chitosan models are colored by green and yellow, respectively, and sialic acids in SGTG models are highlighted in blue. (A,B) A chitosan model with a DP of 10 with two SGTGs that are apart 6 glucosamine residues of chitosan from each other reflecting 15% of DS. The distances from a C1 of a sialic acid in the back SGTG to those in the front one are ca. 3.5 and 4 nm. (C,D) Interaction of CDO-chitosan with DP of 10 (shown in A and B) with a HA trimer. The views from the side (C) and the top (D) of HA. The key amino acids of HA for interaction with sialic acid in the receptor-binding pocket, V135, D190, and Q226, were depicted with red spheres.[23] Two sialic acids in neighboring SGTGs readily fit two sialic acid binding-sites in a HA trimer. (E) A model of CDO-chitosan with a DP of 50. There exist 6 or 7 glucosamine residue of chitosan between two SGTGs, which reflects the DS value around 15%. SGTG side chains protrude in all directions from the chitosan backbone.

concerns about a catastrophic influenza pandemic. Avian influenza viruses preferentially recognize a ligand with α−2,3-sialylgalactose (Neu5Acα2,3Gal) on avian cells, while human viruses preferentially bind α−2,6-sialylgalactose (Neu5Acα2,6Gal) on human cells.[26–30] CDO-chitosan with a Neu5Acα2,3Gal moiety is readily prepared by converting the sialic acid linkage from α−2,6 to α−2,3, and the study on the prevention of avian influenza virus infection by α−2,3-CDO-chitosan, including varying the length of the sialyloligosaccharide side chain, is now ongoing. We expect CDO-chitosan with a Neu5Acα2,3Gal moiety will be an excellent inhibitor of avian influenza virus infection not only in the case of a pandemic in humans but also for protection of poultry from the highly pathogenic avian influenza viruses spreading throughout the world.

Materials and Methods

Viruses. Three strains of human influenza virus, A/New Caledonia/20/99 (H1N1), A/Panama/2007/99 (H3N2), and B/Shanghai/361/2002, were used in this study. Viruses were inoculated in MDCK cells and incubated in Dulbecco's modified Eagle medium (DMEM, Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 2.5 μg/mL purified trypsin (Sigma-Aldrich Co., St. Louis, MO) at 34 °C for 3 days. Culture medium was harvested and stored at −80 °C after centrifugation at 1500*g* for 10 min. Titers of the virus stocks were estimated as described before[31] by indirect immunofluorescent method using MDCK cells and expressed as CIU/mL. Antibodies used were anti-influenza A virus nucleoprotein mouse monoclonal antibody (Serotec, Oxford, UK) followed by FITC-conjugated antimouse IgG goat serum (Medical & Biological Laboratories Co. Ltd., MBL, Nagoya, Japan) for A/New Caledonia, anti-influenza A virus goat polyclonal antibody (Millipore Co., Billerica, MA) followed by FITC-conjugated antigoat IgG rabbit serum (MBL) for A/Panama, and anti-influenza B virus goat polyclonal antibody (Millipore) followed by rhodamin-conjugated antigoat IgG rabbit serum (MBL) for B/Shanghai.

Chitosan. Chitosan with MW 63600 was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), and those with average MW of 3200, 116000, and 970000 were prepared by Yaizu Suisankagaku Industry Co. Ltd. (Yaizu, Japan). They were all obtained by *N*-deacetylation of chitin from marine products. The deacetylation degrees of chitosan with MW 3200, 116000, and 970000 were estimated as 84.6%, 83.8%, and 84.6%, respectively, by the colloid titration method.[32] The chitosan with MW 116000 was further size-fractionated with gel permeation chromatography

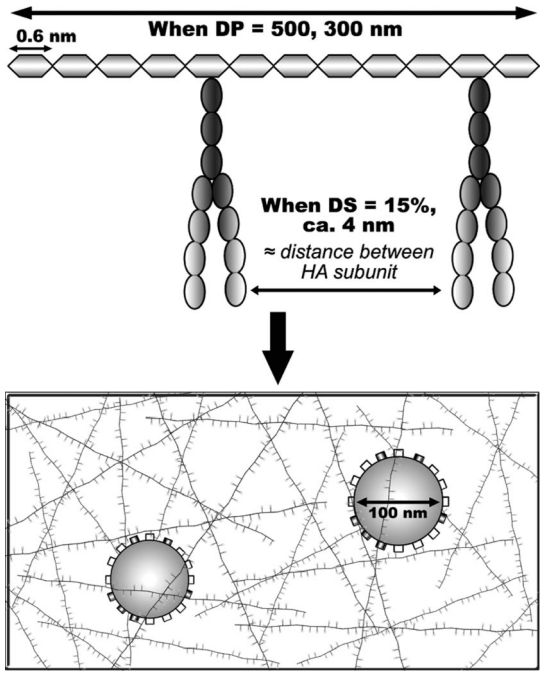

Figure 6. A mesoscopic image of the interactions between influenza viruses and CDO-chitosans. Assuming that the length of a D-glucosamine unit is 0.6 nm, then the length of a chitosan backbone with a DP of 500 is 300 nm. This is three times as long as the virus diameter. When the DS is 15%, the interval between sialic acids in neighboring side chains is ca. 4 nm, which is very close to the distance between the binding pockets of each subunit in trimetric HA.

(column: Toyopearl HW-55F (Tosoh Co., Tokyo, Japan); eluent: 0.1 N HCl containing 0.5% NaCl), because the MW of 116000 was the average and the sample included both smaller and greater MW components than 116000. The three fractions of chitosan with average MW of 231000, 64900, and 18800 were used for the synthesis of the CDO-chitosan. The DP of the chitosan was estimated from the division of the chitosan MW by D-glucosamine MW (162). The estimated DPs were 393, 5990, 1430, 401, 116, and 20 for the chitosans with MW 63600 (for **1a**, **3**), 970000 (for **1b**), 231000 (for **1c**), 64900 (for **1d**, **2a**−**3**), 18800 (for **1e**), and 3200 (for **1f**), respectively.

Synthesis of CDO-Chitosan. The synthesis of CDO-chitosan was previously described in full detail.[6] The procedure is shown in Figure 1. Sialylglycopeptide (SGP), which can be prepared from hen egg yolk,[7] was kindly donated by Taiyo Chemical Industry Co. Ltd. (Yokkaichi, Japan). The saccharide moiety of SGP was transglycosylated onto *p*-FP-GlcNAc utilizing the transglycosylation activity of the recombinant endo-β-*N*-acetylglucosaminidase from *Mucor hiemalis* (Endo-M)[8,9] in 0.1 M sodium phosphate buffer (pH 6.0) at 30 °C for 16 h. The transglycosylation product (SGTG) was obtained using a high-performance liquid chromatography system from Hitachi High-Technologies Co. (Tokyo, Japan) equipped with a UV−vis detector and the column Cosmosil $5C_{18}$-AR-II (Nacalai Tesque, Kyoto, Japan). After evaporating and lyophilization, 106 mg of SGTG (46 μmol) was obtained from 572 mg of SGP (200 μmol) and 248 mg of *p*-FP-GlcNAc (763 μmol) (yield 23%). Chitosan was dissolved with stirring in 1% acetic acid at a concentration of 2 mg/mL, followed by neutralization with 3 M NaOH. The resulting solution (1.2 mg of chitosan containing 7.5 μmol of D-glucosamine in 624 μL) was diluted in a mixture (9 mL) of distilled water and methanol (4:1) with 7.2 mg SGTG (3 μmol). A volume of 10 μL of 1 M sodium cyanoborohydride (Sigma-Aldrich Co.) in methanol was gradually added every 1.5 h (total 100 μmol). After 2 days with stirring at 37 °C, the reductive amination products were ultrafiltrated on MWCO 30K membrane devices (Millipore) and lyophilized. The yields of CDO-chitosan were 3.4, 2.1, 2.6, 3.6, 2.7, and 3.1 mg using chitosans of DP 393, 5990, 1430, 401, 116, and 20, respectively. The DS of SGTG was adjusted by the equivalent molar ratio of SGTG over D-glucosamine residues of chitosan in the reaction mixture, from 80%, 40%, 20%, 10%, to 5%. Their yields using a chitosan with a DP of 401 were 1.5, 2.1, 1.4, 1.4, and 1.4 mg, respectively.

For the synthesis of asialyl-CDO-chitosan, 215 mg of SGP (75 μmol) was desialylated by 400 mU of *Arthrobacter ureafaciens* sialidase (Nacalai Tesque) in 0.1 M sodium carbonate buffer (pH 5.0, 14 mL) at 37 °C for 30 h, then purified by gel-filtration with Sephadex G-25 (GE Healthcare UK Ltd., Buckinghamshire, England) (yield 71%) and used in the same procedure as for CDO-chitosan synthesis. Three milligrams of asialyl-CDO-chitosan was obtained from 1.2 mg chitosan and 5.2 mg asialyl-transglycosylation product (AGTG).

NMR Spectroscopy. The DS of SGTG over D-glucosamine residues in chitosan was determined from the relative integrated values of ^{1}H NMR signal areas between the axial H-3 protons in Neu5Ac and the methyl protons in Neu5Ac from SGTG and *N*-acetyl-D-glucosamine (GlcNAc) derived from both SGTG and chitosan. Because one SGTG has two Neu5Ac and six *N*-acetyl groups, we can estimate the ratio between SGTG and GlcNAc in chitosan, leading to a DS value estimated from the deacetylation degree of chitosan. ^{1}H NMR data of **1d** was (600 MHz, D_2O, RT): δ 7.33 (d, 2H, $J_{2,3} = J_{6,5} =$ 6.22 Hz, H-2 and H-6 of Ph), 7.09 (d, 2H, $J_{3,2} = J_{5,6} =$ 8.31 Hz, H-3 and H-5 of Ph), 5.14 (d, 1H, H-1 of GlcNAc-1), 4.96 (s, 1H, H-1 of Man-4), 4.60 (d, 1H, H-1 of GlcNAc-2), 4.46 (2d, 2H, H-1 of GlcNAc-5,5′), 4.27 (s, 1H, H-2 of Man-3′), 4.13 (d, 1H, $J_{2,3}$ 2.29 Hz, H-2 of Man-4), 4.02 (d, 1H, $J_{2,3}$ 2.02 Hz, H-2 of Man-4′), 2.68 (2dd, 2H, H-3eq of Neu5Ac-7,7′), 2.03, 2.04, 2.05, 2.06, 2.07, 2.08, 2.10 (7s, 21H, AcN) and 1.73 (2t, 2H, $J_{gem} = J_{3ax,4} =$ 12.17 Hz, H-3ax of Neu5Ac-7,7′). Other CDO-chitosans gave similar NMR data.

Inhibitory Assay of Influenza Virus Infection. Inhibitors were dissolved in phosphate-buffered saline (PBS) at a concentration of 1 mg/mL and serially diluted by 1:4, 1:16, 1:64, and 1:256. We used fetuin (Sigma-Aldrich) and 6′-SLN (kindly donated by Japan Tobacco Industry) as control inhibitors. A volume of 50 μL of each diluted inhibitor was mixed with an equal volume of influenza virus solution containing 4.0×10^5 CIU/mL and incubated at 25 °C for 1 h. A volume of 40 μL of each inhibitor−virus mixture was then inoculated in duplicate on a monolayer of MDCK cells in a 96-well plate. After 1 h of adsorption at 37 °C in 5% CO_2, 150 μL of DMEM supplemented with 5 μg/mL of soybean trypsin inhibitor (Sigma-Aldrich) was overlaid. The infected cells were incubated at 37 °C in 5% CO_2 for 14 h and were then fixed by 1% paraformaldehyde in PBS for 1 h, followed by treatment with 1% Triton X-100 for 15 min. After washing with PBS, cells were stained by the indirect immunofluorescent method. Antibodies used for each virus were the same as those used in the virus titration. The positive cells were counted using a fluorescent microscope (Carl Zeiss, Inc., Thornwood, NY) ($N = 4$, two fields per each of two wells). The residual infectivity was expressed as a percentage where the infected cells obtained without inhibitor were set as 100%.

Molecular Modeling. The molecular structures of SGTG[24,33,34] and a chitosan[25] with a DP of 10 were constructed using the Materials Studio (version 3.2, Accelrys Software Inc., San Diego, CA). The chitosan was constructed with only D-glucosamine although chitosans used in experiments included ca. 20% of *N*-acetyl-D-glucosamine. We performed each molecular dymamics simulation of the SGTG and the chitosan in explicit water molecules using the SANDER module in the AMBER 7 program.[35] The Parm99 force-field parameters[36] augmented with the GLYCAM ones[37] (version 06b) were used. The 5 ns calculation was performed for each solute at 298 K and a pressure of 1 atm in *NPT* ensemble, with 2 fs time steps after energy minimization and annealing for water. After the calculation, the solute structures were extracted to construct the CDO-chitosan model. Two simulated SGTG models were conjugated to D-glucosamine in the simulated chitosan model as the interval of the two conjugated residues was 6 to yield the DS of 15%. The contact structure of HA and the CDO-chitosan was made using the Materials Studio software. The CDO-chitosan with a DP of 50 and a DS of 15% was constructed with five chitosan models having a DP of 10 and eight SGTG models to reflect the DS of 15%.

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Supporting Information Available: ^{1}H NMR spectral data of **1d**. This material is available free of charge via the Internet at http://pubs.acs.org.

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