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Inhibition of influenza virus infection with chitosan–sialyloligosaccharides ionic complex

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ABSTRACT

With the recent emergence of drug-resistant influenza viruses, effective means of preventing and treating these contagious pathogens have become imperative. The binding receptors of influenza virus are sialyloligosaccharides (SOS), which are present on the surfaces of host cells, and are therefore attractive targets for antiviral development. We report the preparation and identification of a novel influenza virus entry inhibitor, designated chitosan–SOS complex (CS complex). The CS complex was formed through noncovalent adsorption between cationic chitosan and anionic SOS, the latter derived from bovine colostrum. The preparation was accomplished in gram quantities from chitosan and bovine colostrum oligosaccharides by a one-step dialysis process. The inhibitory activity of the complex against influenza virus infection was determined by cytotoxicity inhibition assay (IC_{50} = 42 μM). This simple preparation, combined with efficient anti-infective activity and the rich natural availability of chitosan and SOS, highlights the potential of the CS complex as a safe, practical agent for influenza prevention and control.

1. Introduction

Influenza is an infectious disease caused by influenza viruses. While influenza prevention and control is primarily implemented through vaccination, universal vaccines that confer protection against all existing subtypes and strains of influenza virus are currently lacking. In addition, the extended time required to produce vaccines (over six months (Gerdil, 2003)) precludes the development of rapid mass vaccinations that would curtail the circulation of emerging viruses. Antiviral medications are indispensable adjuncts to vaccines. However, on account of their high genetic variability, influenza viruses easily establish resistance to currently available drugs (rimantadine, amantadine, oseltamivir, and zanamivir), which has increasingly diminished the effectiveness of these drugs (Bright et al., 2005; Ferraris & Lina, 2008; Tamura et al., 2009). Consequently, alternative means of combating emerging drug-resistant and pandemic viruses have become an urgent priority.

Viral infection begins with attachment of the virus to host cells, which is mediated by the binding of viral hemagglutinin (HA) proteins to carbohydrates containing terminal sialic acid (sialyloligosaccharides, SOS) residing on the host cell surfaces (Imai & Kawaoka, 2012). The specific HA–SOS interaction presents an attractive target for anti-influenza drug designs. Indeed, various SOS mimics that act as virus entry inhibitors have been created in the past (Carlescu, Scutaru, Popa, & Uglea, 2009). Among the most promising drug candidates, are the conjugates containing many copies of SOS linked to appropriate scaffolds such as polymers (Ogata et al., 2007; Oka et al., 2009; Sigal, Mammen, Dahmann, & Whitesides, 1996; Totani et al., 2003; Tsuchida et al., 1998). The inhibitory activity of SOS-conjugates is generally substantially greater than that of monomeric SOS, because multivalent SOS molecules in the SOS-conjugates simultaneously bind to HAs on the virus envelope (glycocluster effect (Collins & Paulson, 2004; Lee & Lee, 1995)). Our group and others have previously developed a class of chitosan conjugates that display SOS branches along the chitosan backbone, and have demonstrated their ability to antagonize influenza virus HA (Li, Wu, Gao, & Cheng, 2011; Makimura et al., 2006; Umemura et al., 2008; Umemura et al., 2010). As this class

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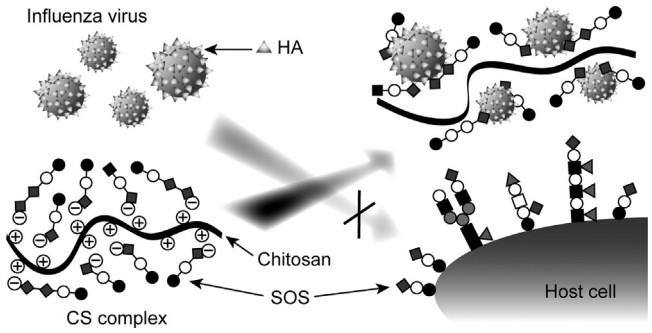

Fig. 1. Inhibition of influenza virus attachment to host cell by CS complex.

of HA inhibitors exploits the unique properties of natural polysaccharide chitosan, such as biocompatibility, biodegradability, and antimicrobial activity (Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004), their safety as potential anti-influenza agents is guaranteed. However, the construction of such chemically conjugated molecules is delicate and arduous, involving multiple synthetic steps, tedious purification procedures, and the use of scarce sialyltransferase and CMP-sialic acid to generate SOS. Consequently, production yields are generally low and practical utility is greatly limited.

In our search for practical and safe anti-influenza agents, we have focused on bovine colostrum, a rich natural source of SOS and other bioactive molecules (Nakamura et al., 2003; Tao et al., 2008). The SOS component mainly includes 3′-sialyllactose (3′SL), 6′-sialyllactose (6′SL), 6′-sialyllactosamine (6′SLN), and disialyllactose (DSL). These molecules are particularly enticing for antimicrobial drug development because they can function as receptor analogs that inhibit infection by many pathogens, including influenza virus (Zivkovic & Barile, 2011). Based on our earlier findings on multivalent SOS–chitosan conjugates (Li et al., 2011), and given the polycationic nature of chitosan that is frequently complexed with nucleic acids for gene delivery (Jayakumar et al., 2010; Mao, Sun, & Kissel, 2010; Muzzarelli, 2010a), we identified chitosan–SOS ionic complex (CS complex) as a simple antiviral alternative to SOC-conjugates (Fig. 1). In the CS complex, the anionic SOS, enriched by the polyglucosamine backbone of chitosan, can fully exploit the glycocluster effect, enabling efficient binding to influenza HA and antagonism against viral attachment to the host. Here, we present the concise preparation of CS complex from bovine colostrum and chitosan, and demonstrate its in vitro effect in treating influenza virus infection.

2. Materials and methods

2.1. Materials

Chitosan was purchased from Yaizu Suisankagaku Industry Co., Ltd. The molecular weight (M_w) of the chitosan, 500 kDa, was measured by gel permeation chromatography (GPC), and its degree of deacetylation (DDA), 80%, was determined by ^{1}H NMR spectroscopy. Colostrum was collected from Holstein cows (1 day after calving) at the dairy farm of the Logistics Department of Lanzou Military Area (Lanzou, China) and stored at −20 °C until use. Standards for thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) analyses were Lactose (Lac), *N*-acetyllactosamine (LN), 3′SL, 6′SL, 6′SLN and DSL, obtained from Sigma–Aldrich Corporation. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) used in the cytotoxicity assay was also obtained from Sigma–Aldrich Corporation. Other reagents were obtained from commercial sources and were of analytical or HPLC grade. Influenza A/PR/8/34 (H1N1) virus was propagated in the allantoic cavity of 10-day-old embryonated chicken eggs. Infected allantoic fluid was pooled and clarified by sucrose-density gradient centrifugation. The hemagglutination unit (HAU) of the purified virus was determined by hemagglutination assay. Madin–Darby bovine kidney (MDBK) cells were grown and maintained in minimum essential medium (MEM) Eagle supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin G and 100 μg/mL streptomycin. The cytotoxicity of influenza virus to MDBK cells was determined by MTT assay (Mosmann, 1983) and expressed as 50% tissue culture infectious dose ($TCID_{50}$).

2.2. Measurements

^{1}H NMR spectra were recorded on a Bruker Avance spectrometer at 500 MHz. Electrospray ionization mass spectra (ESI-MS) were recorded on a Fourier transform mass spectrometer (Bruker Apex IV). The M_w of chitosan was measured by a GPC system (CTO-20A, Shimadzu Co., Ltd.) with a multiangle laser light scattering detector (DAWN HELEOS-II, Wyatt Technology Corp.) in an acetate buffer solution (pH 4.5). HPLC was performed on a Shimadzu Prominence LC-20AT system with a Develosil C30 column (2 × 150 mm) and a UV detector (SPD-M20A). Oligosaccharides were fluorescently labeled with 2-aminopyridine (2-AP) using a glycan labeling kit (Ludger Ltd.) and subsequently purified by gel filtration chromatography (Sephadex G-15 column) prior to HPLC analysis. Dialysis was conducted using a cellulose ester (CE) membrane bag with an MWCO of 300 kDa (Thermo Fisher Scientific Inc.). MTT cytotoxicity assay was performed using a multimode reader (Infinite 200 PRO, Tecan Group Ltd.).

2.3. Preparation of bovine colostrum oligosaccharides (BCO)

Colostrum was defatted and deproteinized as previously reported (Tao et al., 2008). Briefly, the defrosted colostrum (1 L) was centrifuged (2056 × *g*, 30 min) and the top fatty layer was removed. The partially defatted colostrum (820 mL) was thoroughly mixed with 4 volumes of chloroform/methanol (2:1 v/v) followed by centrifugation at 2056 × *g* for 30 min. The upper layer was collected and mixed with 2 volumes of ethanol. The mixture was set aside overnight and then centrifuged (5712 × *g*, 30 min) to remove denatured proteins. Following concentration and lyophilization of the supernatant, BCO (28.2 g) was obtained. The composition of BCO was analyzed by HPLC, ^{1}H NMR and ESI-MS spectroscopies. ^{1}H NMR (D_2O): δ 5.17 (d, J = 3.6 Hz, H-1 of Glc α-isomer), 4.60 (d, J = 8.0 Hz, H-1 of Glc β-isomer), 4.38 (d, J = 7.8 Hz, H-1 of Gal), 3.91–3.45 (m, protons of sugar ring), 3.21 (t, J = 8.0 Hz, H-2 of Glc β-isomer), 1.82 (s, $COCH_3$). The calculated and ESI-MS (m/z) of BCO constituents compare as follows: $C_{12}H_{22}O_{11}$ (Lac): Calcd 342.12. Found 364.81 $[M+Na]^+$; $C_{14}H_{25}NO_{11}$ (LN): Calcd 383.14. Found 405.87 $[M+Na]^+$; $C_{23}H_{39}NO_{19}$ (3′ or 6′SL): Calcd 633.21. Found 655.76 $[M+Na]^+$.

2.4. Isolation of SOS from BCO

The SOS was isolated from BCO by gel filtration chromatography as previously described (Nakamura et al., 2003). Briefly, BCO (28 mg) was dissolved in deionized water (1 mL) and passed through a Bio Gel P-4 (Bio-Rad) column with deionized water as eluent. Fractions were monitored by TLC (2:2:1 acetone/2-propanol/aqueous lactic acid (0.1 M)) using Lac, LN, 3′SL, 6′SL, 6′SLN and DSL as standards. The SOS fractions were pooled and lyophilized, yielding a white solid (1.07 mg, 3.8 wt% BCO). The Lac component (26.65 mg, 95.2 wt% BCO) was also obtained by lyophilization of the corresponding fractions. The SOS composition was analyzed by HPLC, ^{1}H NMR and ESI-MS spectroscopies. ^{1}H NMR

(D_2O): δ 5.13 (d, J = 3.7 Hz, H-1 of Glc α-isomer), 4.57 (d, J = 8.0 Hz, H-1 of Glc β-isomer), 4.41 (d, J = 7.8 Hz, H-1 of Gal), 4.00 (d, J = 9.3 Hz, H-3 of Gal in 3′SL), 3.86–3.39 (m, protons of sugar ring), 3.12 (t, J = 8.4 Hz, H-2 of Glc in 3′SL), 2.68–2.46 (m, H-3eq of Sia), 1.97–1.89 (m, $COCH_3$), 1.62–1.50 (m, H-3ax of Sia). The calculated and ESI-MS (m/z) of SOS constituents compare as follows: $C_{23}H_{39}NO_{19}$ (3′ or 6′SL): Calcd 633.21. Found 656.45 $[M+Na]^+$; $C_{25}H_{42}N_2O_{19}$ (6′SLN): Calcd 674.24. Found 697.82 $[M+Na]^+$; $C_{34}H_{56}N_2O_{27}$ (DSL): Calcd 924.31. Found 947.77 $[M+Na]^+$.

2.5. Preparation of CS complex

A solution of BCO in deionized water (5–15 mL) was added dropwise to a stirred aqueous 0.1 wt% AcOH solution (5 mL) of chitosan (20 mg, 94 μmol of GlcN). The mixture was stirred at room temperature for 3 h and then subjected to dialysis (300 kDa MWCO membrane, deionized water as dialysate). After 4 days (replacing dialysate twice daily), the solution inside the dialysis bag was lyophilized to yield CS complex as a white amorphous solid. BCO of 6.27 g, 4.70 g, 3.13 g, and 1.57 g (with respective Sia content, estimated from 3′SL, of 376 μmol, 282 μmol, 188 μmol, and 94 μmol) afforded CS complexes **1** (66.6 mg), **2** (53.5 mg), **3** (36.6 mg) and **4** (25.1 mg), respectively. In addition, the 16-fold scale-up of preparing the complex **1** yielded 1.02 g of product. ^{1}H NMR (D_2O): δ 5.13 (brs, H-1 of Glc α-isomer), 4.56 (d, J = 8.0 Hz, H-1 of Glc β-isomer), 4.49 (brs, H-1 of GlcN), 4.40 (d, J = 7.7 Hz, H-1 of Gal), 4.30 (brm, H-1 of GlcNAc), 3.97 (brs, H-3 of Gal in 3′SL), 3.82–3.35 (brm, protons of sugar ring), 3.11 (t, J = 8.1 Hz, H-2 of Glc in 3′SL), 2.78 (brs, H-2 of GlcN), 2.65–2.48 (m, H-3eq of Sia), 1.87–1.79 (m, $COCH_3$), 1.60–1.48 (m, H-3ax of Sia).

Dialysis of chitosan (20 mg) mixed with BCO (6.27 g) exceeding 4 days resulted in the diffusion of SOS across the membrane. The dialysate was replaced twice daily throughout the first 4 days. Dialysis was continued up to 7 days with no further replacement of dialysate. Outside of the dialysis bag, the dialysate was lyophilized to yield a sialyloligosaccharide mixture (59 mg), named rSOS to distinguish it from SOS isolated from BCO by gel filtration chromatography. The composition of rSOS was quantitatively analyzed by HPLC.

2.6. Hemagglutination inhibition (HAI) assay

HAI assays were performed as previously described (Li et al., 2011). Briefly, two-fold serial dilutions of CS complex in PBS (25 μL, pH 7.2) were added to 96-well microplates. Influenza A/PR/8/34 virus suspension (in PBS, 4 HAU, 25 μL) was then transferred to each well. Following 1 h incubation at 4 °C, 50 μL of 0.5% (v/v) guinea pig erythrocytes in PBS was added to each well. The hemagglutinations and MIC were recorded after 2 h incubation. SOS, BCO, chitosan, and fetuin were used as controls.

2.7. Cytotoxicity inhibition assay

The inhibition of virus-induced cytotoxicity against MDBK cells was analyzed by the MTT method (Mosmann, 1983; Reuter et al., 1999). Briefly, MDBK cells were grown to a monolayer in 96-well microplates in MEM containing 0.5% bovine serum albumin (BSA). The mixtures of influenza A/PR/8/34 virus (5 $TCID_{50}$, 50 μL) and CS complex (various concentrations, 50 μL) in MEM–BSA medium were added to each well. Following 1 day incubation at 37 °C, cell viability was quantified by MTT assay according to standard procedures. SOS, BCO, chitosan, and ribavirin were used as controls. The effect of all samples on cell viability was also measured.

Table 1
Relative quantities of oligosaccharides in BCO, SOS, and rSOS.[a]

Sugars	Sequences	BCO[a,b]	SOS[a,c]	rSOS[a]
Lac	Galβ1,4Glc	71.2	0.8	0.6
LN	Galβ1,4GlcNAc	2.2	0.0	0.0
6′SL	Siaα2,6Galβ1,4Glc	2.6	8.0	9.8
3′SL	Siaα2,3Galβ1,4Glc	20.6	76.2	74.5
6′SLN	Siaα2,6Galβ1,4GlcNAc	3.4	7.2	8.8
DSL	Siaα2,8Siaα2,3Galβ1,4Glc	0.0	7.8	6.3

[a] Mole percent (mol%) of individual oligosaccharide, relative to the sum of all oligosaccharides present in the mixtures (determined by HPLC, see the Supplementary data).
[b] The total concentration of BCO in bovine colostrum is 28.2 g/L (see Section 2.3).
[c] The total concentration of SOS in bovine colostrum is 1.07 g/L, based on the isolated yield of SOS from BCO (see Section 2.4).

3. Results and discussion

3.1. Purification and characterization of SOS

The gross composition of bovine milk, excluding water and minerals, is carbohydrate, fat, and protein. The carbohydrate includes Lac, and various neutral and acidic oligosaccharides. Although the SOS concentration in mature milk is low, it elevates during the early stage of lactation; thus, bovine colostrum is an important natural source of SOS (Nakamura et al., 2003; Tao et al., 2008). To validate the SOS content in the colostrum used in this study, we removed fat and protein from the colostrum using standard procedures (Tao et al., 2008), and designated the resulting carbohydrate mixture as BCO. Along with the predominant Lac, ^{1}H NMR and mass spectroscopies detected several SOS species in the BCO (Fig. 2). To enable precise qualitative and quantitative HPLC analyses (see the Supplementary data), we then purified SOS from BCO by removing Lac and LN through gel filtration chromatography. Four typical structures of SOS, 6′SL, 3′SL, 6′SLN, and DSL, were identified and their relative concentrations were determined (Table 1). Previous analyses using high accuracy mass techniques have revealed over twenty SOS structures in BCO (Tao et al., 2008). Here, although fewer structures were characterized, we confirmed that SOS is abundant in the colostrum (1.07 g/L) and is dominated by 3′SL (76.2 mol% of total SOS). As the anti-influenza activity of SOS, especially 3′SL, has been previously demonstrated (Carlescu et al., 2009), these results encouraged us to proceed with the preparation and bioevaluation of CS complex.

3.2. Preparation and characterization of CS complex

To simplify the procedures, CS complex was prepared from BCO rather than from SOS. Isolation of SOS from BCO requires arduous chromatographic techniques such as HPLC, ion exchange, and gel filtration that are unsuitable for mass production. On the other hand, CS complexes can be generated in large quantities from BCO and cationic chitosan without pre-purification of SOS. CS complexes **1**–**4** (Table 2) were directly prepared from an aqueous solution of chitosan and BCO by a simple dialysis technique. The SOS content in the complexes, represented by the S/N molar ratio, was experimentally adjusted by the BCO quantity relative to chitosan. The maximum SOS density was obtained in CS complex **1** (S/N ratio = 62%). All CS complexes were characterized by ^{1}H NMR spectroscopy (Fig. 3). Their structural compositions were verified by identifying several baseline-separated signals of SOS and chitosan backbone in the NMR spectra. The S/N values were determined by the integral ratio of peaks characteristic of SOS to those characteristic of chitosan. Using the dialysis method, we readily obtained gram quantities of the CS complex **1**.

A crucial parameter in preparing CS complexes is the dialysis time. Most of the neutral carbohydrates (dominated by Lac) were

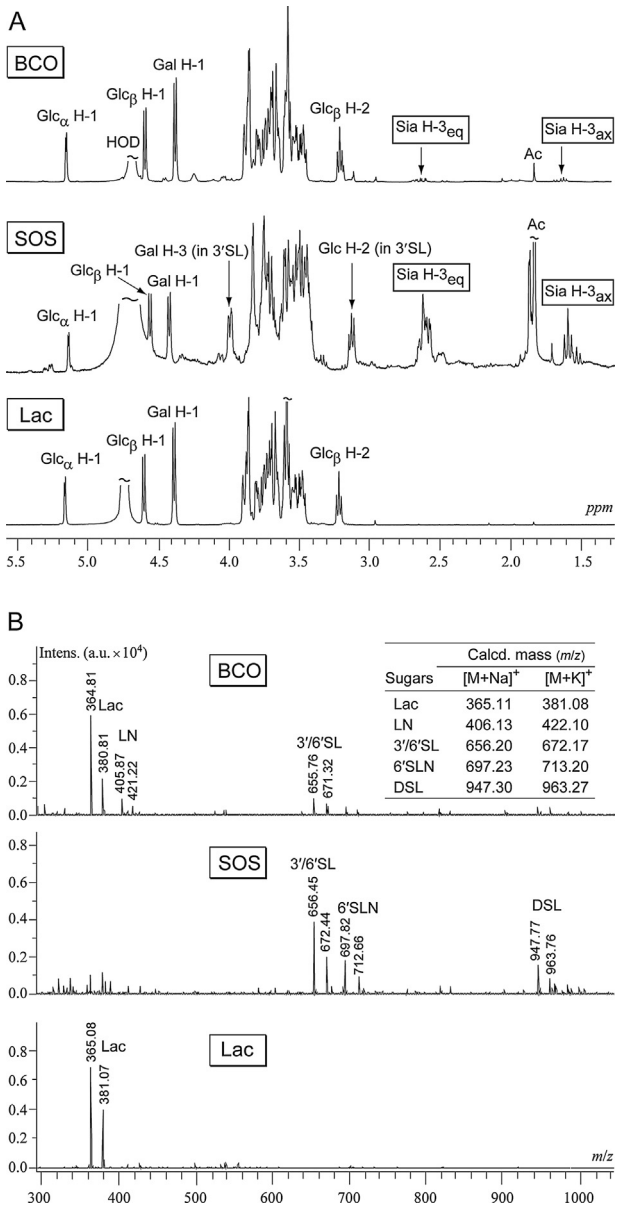

Fig. 2. ^{1}H NMR (A) and ESI-MS (B) spectra of BCO, SOS, and Lac. The BCO were prepared from bovine colostrum with fat and protein pre-removed. The SOS and Lac were isolated from the BCO by gel filtration chromatography. The NMR signals characteristic of sialic acid residues are indicated in rectangle. The mass peaks of the identified carbohydrates are labeled. The NMR spectra were recorded in D_2O, at 500 MHz and 27 °C.

Table 2
CS complexes with different SOS content.

CS complexes	S/N mole ratio[a]	S/C weight ratio[b]
1	0.62	2.40
2	0.45	1.74
3	0.23	0.89
4	0.08	0.31

[a] The S/N mole ratios (ratio of Sia residues to amino groups of chitosan) were determined by the ^{1}H NMR integration ratios of Sia-characteristic peaks to chitosan-characteristic peaks (Sia H-3eq or H-3ax to GlcN H-2; see Fig. 3).

[b] The S/C weight ratios (ratio of SOS to chitosan) were estimated from the S/N mole ratios and the relative quantity (mol%) of oligosaccharides present in SOS (see Table 1).

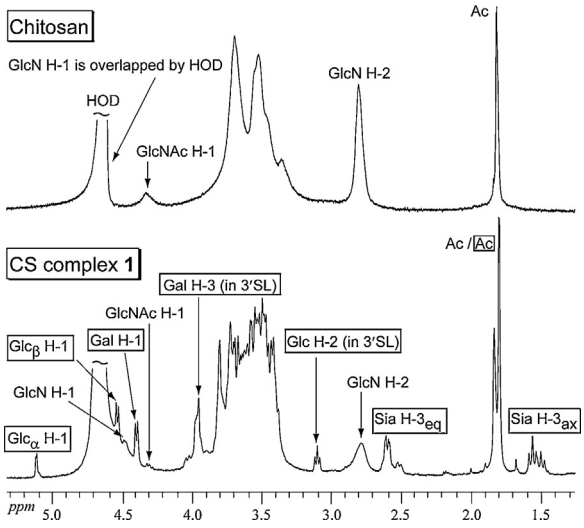

Fig. 3. ^{1}H NMR spectra (500 MHz, 27 °C) of chitosan (in DCl/D_2O) and CS complex **1** (in D_2O). The signals are assigned to chitosan and SOS (labeled in rectangle). The S/N ratio of the CS complex was determined from the area ratio of the Sia H-3eq peak to the chitosan GlcN H-2 peak.

Table 3
Inhibition of influenza virus-induced hemagglutination of erythrocytes by CS complexes.[a]

Smaples	S/N mole ratio	MIC (μg/mL)[b]
CS complex **1**	0.62	42 (45 μM)
CS complex **2**	0.45	85 (82 μM)
CS complex **3**	0.23	138 (99 μM)
CS complex **4**[c]	0.08	NA[d]
SOS	–	NA[d]
BCO	–	NA[d]
Chitosan[c]	–	NA[d]
Fetuin[e]	–	156 (31 μM)

[a] Influenza A/PR/8/34 (H1N1) virus was used.

[b] Minimum inhibitory concentration: μg/mL of sample (μM of SOS). The molarities of SOS were based on the S/C weight ratios (see Table 2) and the relative quantity (mol%) of oligosaccharides present in SOS (see Table 1).

[c] CS complex **4** and chitosan were suspended, but others were dissolved in PBS.

[d] No detectable activity at 1 mg/mL (for SOS, equal to 1.5 mM; for complex **4**, equal to 360 μM of SOS) or less.

[e] On average, one molecule of fetuin ($M_w \approx 50$ kDa) contains approximately 10 Sia-terminated oligosaccharide branches (Baenziger & Fiete, 1979).

removed during the first 4 days of dialysis, while chitosan and SOS were retained inside the dialysis bag, where they formed CS complexes by lyophilization. When the dialysis time was extended (beyond 4 days), the SOS diffused across the dialysis membrane, and was recoverable from the dialysate outside the bag. The content of the recovered SOS (designated rSOS) was nearly identical to the SOS directly isolated from BCO by gel filtration chromatography (Table 1), confirming the structural variety of SOS in the CS complexes. In contrast to water-insoluble chitosan, CS complexes **1**–**3** demonstrated high aqueous solubility (≥1.0 mg/mL, pH 7.2) owing to their high levels of acidic SOS, but complex **4**, with a lower S/N ratio, was less soluble.

3.3. *Inhibition of influenza virus attachment to host cells by CS complex*

The inhibition of CS complex on the viral adhesion to host erythrocytes was evaluated by HAI assay (Table 3). The virus was a laboratory standard H1N1 influenza strain, A/Puerto Rico/8/34 (H1N1), which has known binding specificity for SOS including 3′SL, 6′SL, and 6′SLN (Li et al., 2011; Ogata et al., 2007; Totani et al., 2003). In the presence of complexes **1**–**3**, erythrocyte agglutination was

Table 4
Inhibition of influenza virus-induced cytotoxicity by CS complexes.[a]

Samples	S/N mole ratio	IC_{50} (μg/mL)[b] ± SD[c]
CS complex **1**	0.62	39 (42 μM) ± 0.8
CS complex **2**	0.45	92 (89 μM) ± 2.8
CS complex **3**	0.23	152 (108 μM) ± 3.7
CS complex **4**[d]	0.08	NA[e]
SOS	–	NA[e]
BCO	–	NA[e]
Chitosan[d]	–	NA[e]
Ribavirin	–	0.24 ± 0.2 (0.97 μM)

[a] MDBK cells were incubated with the samples and influenza A/PR/8/34 (H1N1) virus.
[b] Half maximal inhibitory concentration: μg/mL of sample (μM of SOS or ribavirin). The molarities were based on the S/C weight ratios (see Table 2) and the relative quantity (mol%) of oligosaccharides present in SOS (see Table 1).
[c] Standard deviations from three independent experiments.
[d] CS complex **4** and chitosan were suspended, but others were dissolved in MEM-BSA medium.
[e] No observable activity at 1 mg/mL (for SOS, equal to 1.5 mM; for complex **4**, equal to 360 μM of SOS) or less.

inhibited, indicating limited viral attachment. In contrast, chitosan exerted no noticeable anti-adhesion activity, verifying that the SOS component in the complexes was responsible for the observed specific inhibition. The effective inhibition by the complexes suggests that viral attachments to host cells are antagonized by potent binding of chitosan-enriched SOS to the HAs of influenza viruses. In addition, the MIC value of the complexes strongly depends on SOS density. The inhibitory activity increased as the S/N ratio increased; in contrast to complexes **1**–**3**, complex **4** and free SOS showed no detectable activity at concentrations of 1 mg/mL or less. This phenomenon is due to the cluster glycoside effect (glycoconjugates with high densities of sugar moiety frequently bind strongly to their target proteins (Collins & Paulson, 2004; Lee & Lee, 1995)). A number of synthetic molecules containing multivalent Sia, SL, or SLN units are effective influenza virus HA inhibitors (Makimura et al., 2006; Ogata et al., 2007; Oka et al., 2009; Sigal et al., 1996; Totani et al., 2003; Tsuchida et al., 1998; Umemura et al., 2008, 2010). In the present study, the inhibitory efficiency of complex **1** (MIC = 45 μM) is comparable with that of a previously reported SOS–chitosan conjugate (MIC = 54 μM) (Li et al., 2011) as well as fetuin (a naturally occurring sialoglycoprotein (Baenziger & Fiete, 1979)). This result highlights the potential of CS complex **1** as a new HA-binding inhibitor for the treatment of influenza infections.

3.4. Neutralization of influenza virus-induced cytotoxicity by CS complex

Next, the extent to which CS complexes can protect host cells from virus-induced cytopathic effects was determined by cytotoxicity inhibition assay (Table 4). The influenza virus-sensitive MDBK cells incubated with CS complexes **1**–**3** were effectively protected against the lethal effects of influenza virus A/Puerto Rico/8/34. Consistent with the results of the HAI study, the capacity of the CS complex to neutralize viral toxicity depends on the S/N ratio. Complex **1** is the most potent inhibitor among the tested samples, indicating that effective protection of MDBK cells is mediated by the highly enriched SOS molecules that bind HAs through the glycocluster effect. Although the neutralizing potency (IC_{50} = 42 μM) of complex **1** is considerably lower than that of ribavirin, a clinically used nucleoside inhibitor with broad-spectrum antiviral activity, this complex suggests a novel type of entry inhibitor in the treatment of influenza infection. Additionally, MDBK cells exposed to negative controls (chitosan, free SOS, or BCO) or to complex **4** (with a much lower S/N mole ratio) were not effectively protected at the tested concentration. None of the treatments affected cell viability in the absence of influenza virus (data not shown).

4. Conclusions

We have developed a novel class of influenza virus HA inhibitors, designated CS complexes, and have demonstrated their therapeutic potential against influenza infection. Unlike conventional synthetic inhibitors, in which multivalent SOS are chemically conjugated to scaffold molecules, CS complexes are generated by noncovalent electrostatic attraction between cationic chitosan and anionic SOS. This permits the large-scale preparation of CS complex from chitosan and BCO by a one-step dialysis process. The inhibitory activity of CS complex against influenza virus is comparable with that of chitosan-based synthetic inhibitors (Li et al., 2011). Chitosan and BCO are both readily available from natural sources (Kumar et al., 2004; Muzzarelli, 2010b; Zivkovic & Barile, 2011), thereby providing therapeutic benefits in terms of safety and cost-effectiveness (whey permeate from cheese manufacture is recently regarded as a potential low-cost source for industrial production of SOS (Barile et al., 2009)). In particular, as influenza virus binds to a spectrum of SOS structures in the natural hosts (Imai & Kawaoka, 2012), the enriched SOS components in CS complex, with their structural variety and specificity, further enhance the potential of CS complex as a viable anti-influenza agent.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.02.048.

References

Baenziger, J. U., & Fiete, D. (1979). Structure of the complex oligosaccharides of fetuin. *The Journal of Biological Chemistry*, *254*, 789–795.
Barile, D., Tao, N., Lebrilla, C. B., Coisson, J. D., Arlorio, M., & German, J. B. (2009). Permeate from cheese whey ultrafiltration is a source of milk oligosaccharides. *International Dairy Journal*, *19*, 524–530.
Bright, R. A., Medina, M., Xu, X., Perez-Oronoz, G., Wallis, T. R., Davis, X. M., et al. (2005). Incidence of adamantane resistance among influenza A (H3N2) viruses isolated worldwide from 1994 to 2005: A cause for concern. *Lancet*, *366*, 1175–1181.
Carlescu, I., Scutaru, D., Popa, M., & Uglea, C. V. (2009). Synthetic sialic-acid-containing polyvalent antiviral inhibitors. *Medicinal Chemistry Research*, *18*, 477–494.
Collins, B. E., & Paulson, J. C. (2004). Cell surface biology mediated by low affinity multivalent protein–glycan interactions. *Current Opinion in Chemical Biology*, *8*, 617–625.
Ferraris, O., & Lina, B. (2008). Mutations of neuraminidase implicated in neuraminidase inhibitors resistance. *Journal of Clinical Virology*, *41*, 13–19.
Gerdil, C. (2003). The annual production cycle for influenza vaccine. *Vaccine*, *21*, 1776–1779.
Imai, M., & Kawaoka, Y. (2012). The role of receptor binding specificity in interspecies transmission of influenza viruses. *Current Opinion in Virology*, *2*, 160–167.
Jayakumar, R., Chennazhi, K. P., Muzzarelli, R. A. A., Tamura, H., Nair, S. V., & Selvamurugan, N. (2010). Chitosan conjugated DNA nanoparticles in gene therapy. *Carbohydrate Polymers*, *79*, 1–8.
Kumar, M. N., Muzzarelli, R. A. A., Muzzarelli, C., Sashiwa, H., & Domb, A. J. (2004). Chitosan chemistry and pharmaceutical perspectives. *Chemical Reviews*, *104*, 6017–6084.
Lee, Y. C., & Lee, R. T. (1995). Carbohydrate–protein interactions: Basis of glycobiology. *Accounts of Chemical Research*, *28*, 321–327.
Li, X., Wu, P., Gao, G. F., & Cheng, S. (2011). Carbohydrate-functionalized chitosan fiber for influenza virus capture. *Biomacromolecules*, *12*, 3962–3969.

Makimura, Y., Watanabe, S., Suzuki, T., Suzuki, Y., Ishida, H., Kiso, M., et al. (2006). Chemoenzymatic synthesis and application of a sialoglycopolymer with a chitosan backbone as a potent inhibitor of human influenza virus hemagglutination. *Carbohydrate Research*, *341*, 1803–1808.

Mao, S., Sun, W., & Kissel, T. (2010). Chitosan-based formulations for delivery of DNA and siRNA. *Advanced Drug Delivery Reviews*, *62*, 12–27.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, *65*, 55–63.

Muzzarelli, R. A. A. (2010a). Chitosans: new vectors for gene therapy. In R. Ito, & Y. Matsuo (Eds.), *Handbook of carbohydrate polymers: Development, properties and applications* (pp. 583–604). New York: Nova Science Publishers Inc.

Muzzarelli, R. A. A. (2010b). Chitins and chitosans as immunoadjuvants and non-allergenic drug carriers. *Marine Drugs*, *8*, 292–312.

Nakamura, T., Kawase, H., Kimura, K., Watanabe, Y., Ohtani, M., Arai, I., et al. (2003). Concentrations of sialyloligosaccharides in bovine colostrum and milk during the prepartum and early lactation. *Journal of Dairy Science*, *86*, 1315–1320.

Ogata, M., Murata, T., Murakami, K., Suzuki, T., Hidari, K. I., Suzuki, Y., et al. (2007). Chemoenzymatic synthesis of artificial glycopolypeptides containing multivalent sialyloligosaccharides with a gamma-polyglutamic acid backbone and their effect on inhibition of infection by influenza viruses. *Bioorganic & Medicinal Chemistry*, *15*, 1383–1393.

Oka, H., Onaga, T., Koyama, T., Guo, C. T., Suzuki, Y., Esumi, Y., et al. (2009). Syntheses and biological evaluations of carbosilane dendrimers uniformly functionalized with sialyl α(2⟶3) lactose moieties as inhibitors for human influenza viruses. *Bioorganic & Medicinal Chemistry*, *17*, 5465–5475.

Reuter, J. D., Myc, A., Hayes, M. M., Gan, Z., Roy, R., Qin, D., et al. (1999). Inhibition of viral adhesion and infection by sialic-acid-conjugated dendritic polymers. *Bioconjugate Chemistry*, *10*, 271–278.

Sigal, G. B., Mammen, M., Dahmann, G., & Whitesides, G. M. (1996). Polyacrylamides bearing pendant α-sialoside groups strongly inhibit agglutination of erythrocytes by influenza virus: The strong inhibition reflects enhanced binding through cooperative polyvalent interactions. *Journal of the American Chemical Society*, *118*, 3789–3800.

Tamura, D., Mitamura, K., Yamazaki, M., Fujino, M., Nirasawa, M., Kimura, K., et al. (2009). Oseltamivir-resistant influenza a viruses circulating in Japan. *Journal of Clinical Microbiology*, *47*, 1424–1427.

Tao, N., DePeters, E. J., Freeman, S., German, J. B., Grimm, R., & Lebrilla, C. B. (2008). Bovine milk glycome. *Journal of Dairy Science*, *91*, 3768–3778.

Totani, K., Kubota, T., Kuroda, T., Murata, T., Hidari, K. I., Suzuki, T., et al. (2003). Chemoenzymatic synthesis and application of glycopolymers containing multivalent sialyloligosaccharides with a poly(L-glutamic acid) backbone for inhibition of infection by influenza viruses. *Glycobiology*, *13*, 315–326.

Tsuchida, A., Kobayashi, K., Matsubara, N., Muramatsu, T., Suzuki, T., & Suzuki, Y. (1998). Simple synthesis of sialyllactose-carrying polystyrene and its binding with influenza virus. *Glycoconjugate Journal*, *15*, 1047–1054.

Umemura, M., Itoh, M., Makimura, Y., Yamazaki, K., Umekawa, M., Masui, A., et al. (2008). Design of a sialylglycopolymer with a chitosan backbone having efficient inhibitory activity against influenza virus infection. *Journal of Medicinal Chemistry*, *51*, 4496–4503.

Umemura, M., Makimura, Y., Itoh, M., Yamamoto, T., Mine, T., Mitani, S., et al. (2010). One-step synthesis of efficient binding-inhibitor for influenza virus through multiple addition of sialyloligosaccharides on chitosan. *Carbohydrate Polymers*, *81*, 330–334.

Zivkovic, A. M., & Barile, D. (2011). Bovine milk as a source of functional oligosaccharides for improving human health. *Advances in Nutrition*, *2*, 284–289.