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Graphical Abstract

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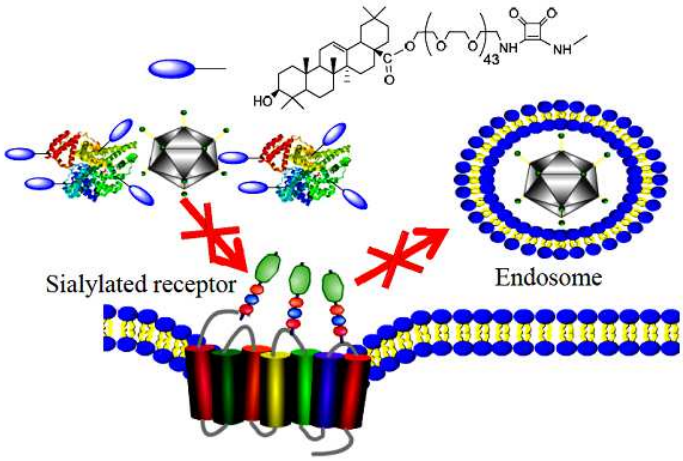

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Multivalent oleanolic acid human serum albumin conjugate as nonglycosylated neomucin for influenza virus capture and entry inhibition

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ABSTRACT

We report the synthesis of multivalent oleanolic acid (OA) protein conjugates as nonglycosylated neomucin mimic for the capture and entry inhibition of influenza viruses. Oleanolic acid derivatives bearing an amine-terminated linker were synthesized by esterification of carboxylic acid and further grafted onto the human serum albumin (HSA) *via* diethyl squarate method. The binding of hemagglutinin (HA) on the virion surface to the synthetic neomucin was evaluated by hemagglutination inhibition assay. The influenza virus capture ability of the PEGylated OA-HSA conjugate was further investigated by Dynamic Light Scattering (DLS), virus capture assay and Isothermal Titration Calorimeter (ITC) techniques. The pronounced agglutination of viral particles, the high capture efficiency and affinity constant indicate that this neoprotein is comparable to natural glycosylated mucin, suggesting that this material could potentially be used as anti-infective barriers to prevent virus from invading host cells. The study also rationalizes the feasibility of antiviral drug development based on OA or other antiviral small molecules conjugated protein strategies.

1. Introduction

Influenza virus is a deadly respiratory virus capable of causing significant harm to the population. For example, recent outbreaks of avian H7N9 influenza in South China have caused 79 deaths and 192 hospitalizations in January 2017[1]. This outbreak is far from over as new cases of infection and mortality are being reported[2]. Currently, Zanamivir (Renlenza®) and Osetamivir (Tamiflue®) as neuraminidase inhibitors (NAIs), which can competitively occupy the active site of the NA and disturbing virus propagation[3,4] have been approved in many countries and used for the treatment of H7N9 virus infections. However, resistance to these drugs have emerged with two strains containing the NAIs resistant mutations in this 2017 pandemic as reported by China CDC and WHO [5]. Although the resistant strains could be managed by oral administration of large dose of Tamiflue®[6], the side effects can be significant. Additionally, the constant emergence of NAIs-resistant viruses in humans emphasizes the need for newer methods of prevention and treatment. In fact, China CDC warrants continuous surveillance of live poultry markets for the appearance of pathogenic avian influenza since 2017 [7]. Also, novel methods and strategies are required for the development of new antiviral agents targeting different steps in the viral life cycle for resistant strains [8-10].

In the past few years, a new strategy inspired by mucin[11], a key proteoglycan component of the mucosal layer covering epithelial cells in many tissues of humans and animals as part of defense systems, has been considered in the generation of new antiviral conjugates[12]. Although the intricate mechanistic details by which mucin prevents influenza from infection is still under investigation, it is generally accepted that these highly glycosylated protein with large molecular weight (>100 kD) can block the virial hemagglutinin (HA)-based binding [13] to the host

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cell and neuramidinase (NA)-based releasing[14] from the infected cell *via* multivalent sialic acid (SA)-HA/NA interactions. Researchers have attempted to mimic Nature by attaching multiple copies of SA or SA analogs to polymers, dendrimers, liposome and even metal nanoparticles as effective antiviral barrier for anti-influenza drug development [15] [16]. Some of the studies have shown promise under various assay conditions. However, there are two drawbacks to these multivalent glycoconjugates. First, the natural *O*-linked sialosides could be hydrolyzed by NA[17] leading to the decrease of the binding activity. To overcome this problem, studies using pseudo (S, N and C) sialyl α-2,3 or α-2,6 galactosylsaccharide have been synthesized [18] and conjugated onto different scaffolds [19,20], which are stable towards NA cleavage. A second and more pressing concern is that natural and modified SA conjugates are not synthesis friendly, as numbers of protection/deprotection strategies with multiple purification steps are required. Chemoenzymatic strategies for the synthesis of complex carbohydrates are gaining more recognition[21-23], but the engineering enzymes and improving susbstrate specificity, etc. are also labor and time intensive. While carbohydrate based drug development will continue to be a major force in the near and distant future, developing novel nonglycosylated influenza inhibitors, which are relatively easy to synthesize is highly desirable. Here, we report our initial studies with a naturally occurring compound, oleanolic acid (OA) that functions as a broad spectrum entry inhibitor of influenza viruses.

OA belongs to pentacyclic triterpene family, with only one carboxyl and hydroxyl group has been recently reported to have tighter binding to HA with disassociation constant K_D around micromolar range, which is much higher than monomeric SA with millimolar K_D [24]. Conjugation of OA onto cyclodextrin [25,26] has been shown to significantly enhanced its influenza entry inhibitory activity. Encouraged by the dramatic activity enhancement induced by the cluster effect [27,28], we designed design and prepare OA protein conjugates as multivalent nonglycosylated neomucin for the development of influenza capture and entry inhibitor. Specifically, natural protein presents in plasma (Human Serum Albumin, HSA) was adopted as the artificial mucin backbone because of its availability, biodegradability, nontoxicity and nonimmunogenicity, which is well established in our previous work[29]. The molecular weight of this new neomucin was easily characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)[30], which makes it feasible to quantitatively measure the virus interactions. Finally, the virus capture and entry inhibitory ability of the synthetic nonglycosylated neomucin was demonstrated.

2. Results and discussion

2.1.Chemistry

The syntheses of the HSA-OA conjugates are summarized in Schemes 1. To realize covalent coupling between OA and HSA, azide as the precursor of amine was first introduced into the 28-position of **OA**. Esterification of **OA** with 1-azido-6-bromohexane or 1-azido-2-(2-(2-bromoethoxy)ethoxy)ethane gave azide, **C6-OA**, **2** (42%) and **OEG-OA**, **3** (45%) , respectively[31,32]. After reducing the azide with Lindlar catalyst under hydrogen atmosphere, the resultant amine was first reacted with one of the esters of squaric acid diethyl esters in potassium phosphate buffer (pH 7.0) to give **5** and **6**, respectively. After purification with column chromatography, the remaining ester group of **5** or **6** was coupled with the amines on the lysine residue side chains of HSA in borate buffer (pH 9.0) to obtain the conjugates **HSA-C6-OA** or **HSA-OEG-OA** [29,33]. The number of OA derivatives attached onto protein backbone was determined by MALDI-TOF-MS (see Supplementary Material). Mass analysis of **5** or **6** revealed that about only 2 or 3 OA molecules were attached onto one molecule of HSA (Table 1). The relative insolubility and steric hindrance of the functional ligands **5** and **6** may affect the coupling efficiency.

To improve the hydrophilicity and chain flexibility, we chose poly (ethylene glycol) (PEG), which has been widely used in protein chemistry for crosslinking[34,35] as the linker for the HSA conjugation. An uncapped 2,000 average molecular weight PEG diol **HO-PEG-OH** was selected and subjected to OA functionalization. Mono tosylation to **HO-PEG2000-OH** can be achieved by using Ag_2O/KI [36]. The resulting monotosylate was converted to amine **8** *via* ammonolysis with ammonia. This product was subsequently reacted with $(Boc)_2O$ to give amide **9**. Repeating the tosylation at the other end of the alcohol and substitution of tosylate with LiBr gave the corresponding bromide **10** with a yield of 85% [37]. According to the established esterification method, **PEG2000-OA**, **4** was successfully synthesized in 97% yield. After treatment of **4** with TFA, a similar squaric acid diethyl ester procedure was applied for the preparation of PEGylated OA decorated HSA. After dialysis, purification on Sephadex G-75® and lyophilization, the HSA-OA conjugates were characterized by MALDI-TOF mass spectrometry again. Mass analysis of **HSA-PEG2000-OA** conjugate showed that an average number of 5 **PEG2000-OA** was attached per one molecule of HSA. Compared with corresponding parent OA, all of the HSA-OA conjugates had high solubility in PBS, indicating its further application in bioassay system. Methoxypolyethylene glycols modified HSA (**HSA-PEG2000-OMe)** was also prepared as negative control.

Table 1. Conjugation of OA derivatives with HSA

Entry	Molecular Weight[a]	Loading OA residues
HSA	66740.67	0
HSA-PEG2000-OMe	79812.97	0/6[b]
HSA-C6- OA	67820.62	2
HSA-OEG-OA	69147.45	3
HSA-PEG2000- OA	80251.87	5

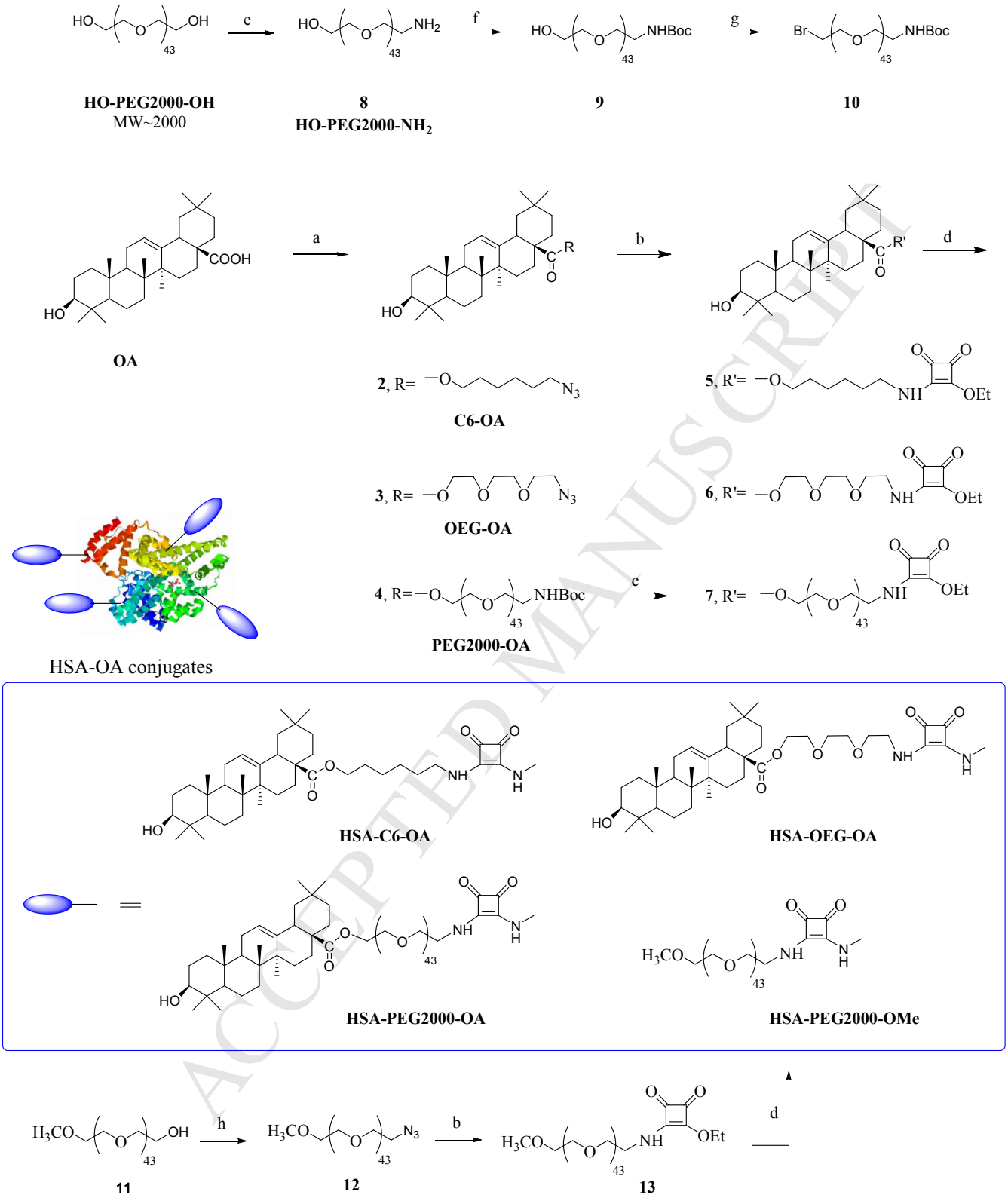
[a] Molecular weight was determined by MALDI-TOF

[b] Number of molecules of the **PEG2000OMe** derivative, **13** attached to HSA

2.2. Bioassay

2.2.1. HAI assay

An easy-to-perform hemagglutination inhibition (HAI) assay [38] was initially applied for evaluating the binding of the influenza virus to the synthetic HSA-OA conjugates. We choose three different virus strains that have different HA subtypes to demonstrate broad specificity. Table 2 showed the results of K*i* [39], which defined as the lowest concentration of the OA derivatives that prevented hemagglutination, using human influenza virus [A/Puerto Rico/8/1934 (H1N1), A/Huairou, Beijing/11069/2014 (H3N2)] or chicken influenza virus [A/Chicken/Beijing/AT609/2014 (H9N2)] as the sources of HA. It is evident that all the HSA-OA conjugates bound influenza virus more strongly than HSA and **HSA-PEG2000-OMe**, which doesn't possess OAs. Compared with OA monomer, the lower K_i of the conjugates indicated the cluster effect [27,28] induced by the covalent attachment of multiple OA residues onto the HSA backbone. Moreover, **HSA-PEG2000-OA** was the best agglutinating conjugate; its binding efficiency is comparable with mucin, a naturally occurring protein from bovine submaxillary glands (BSG, MW ~ 400 kDa), which has been used as a control to define the potency of HA inhibitor[40]. The decreased K_i value of **HSA-PEG2000-OA** also suggested that the PEGylation



Scheme 1. Synthesis of **HSA-OA** conjugates. (a) K_2CO_3, bromide linker; (b) (i) H_2, Lindlar catalyst (ii) squaric acid diethyl esters, phosphate buffer saline (pH 7.0); (c) (i) TFA/CH_2Cl_2 (ii) squaric acid diethyl esters, phosphate buffer saline (pH 7.0); (d) HSA, borate buffer buffer (pH 9.0); (e) (i) Ag_2O, KI, TosCl (ii) $NH_3{\cdot}H_2O$, NH_4Cl; (f) $(Boc)_2O$, Et_3N, CH_2Cl_2; (g) (i) TosCl, Et_3N, CH_2Cl_2 (ii) LiBr/acetone, overnight; (h) (i) TosCl, Et_3N, CH_2Cl_2 (ii) NaN_3/DMF, overnight.

is a useful modification strategy for improving the biocompatibility and bioactive compound efficacy.

2.2.2. Dynamic Light Scattering, DLS

The successful synthesis and influenza binding bioactivity verifications of biocompatible **HSA-PEG2000-OA** encouraged us to further study the capturing ability of **HSA-PEG2000-OA**.

Table 2. Inhibition constant Ki of HAI assay

Entry	Ki (μM)		
	H1N1	H3N2	H9N2
HSA	125	125	125
HSA-PEG2000-OMe	NA	62.5	62.5
OA	60	60	30
HSA-C6-OA	108	54	54
HSA-OEG-OA	54	13.5	13.5
HSA-PEG2000-OA	3	3	3
Mucin	0.16	0.16	0.16

Virus capture behavior of the **HSA-PEG2000-OA** was thoroughly investigated by Dynamic Light Scattering, a technique that can provide a quantitative estimate of the average diameter of the aggregates induced by the adsorption of numerous virions with HSA-OA conjugate [41,42]. From the DLS measurements, the size of the HSA conjugates and viral particles were found to be around 15 nm and 120 nm, respectively. After incubation with virus for 1 h, the mean hydrodynamic diameters of **HSA-PEG2000-OA** had increased to ~ 300 nm and the peak of the virus almost disappeared, clearly indicating aggregate formation (Figure 1). However, no aggregation was observed after the addition of the virus to **HSA-PEG2000-OMe** solution, as no larger standard deviation was measured. The DLS peaks clearly showed our HSA-OA conjugate could cross-link several virus particles and could be a promising capture biomacromolecule for influenza inhibition and detection development.

2.2.3. Virus capture assay

To further examine the virus-capture capability of **HSA-PEG2000-OA**, a simple assay was applied using the solution mixture after DLS measurement. The mixture in the sample cell for the DLS analysis was centrifuged at 3000 rpm for 5 min. The supernatant was added into black 384-well plate and 25 μL MUNANA [2´-(4-Methylumbelliferyl)-α-*D*-N-acetylneuraminic acid] in 0.1 M sodium acetate buffer (pH 5.5) containing 10 mM $CaCl_2$ was added. The hydrolysis of MUNANA by neuraminidase (NA) on the virial surface producing the fluorescent signal was monitored for the NA activity[43, 44]. The NA activity in the absence of HSA conjugates was defined as 100% and in the presence of **HSA-PEG2000-OMe** was used as negative control, respectively.

As shown in Table 3, the virus activity for H1N1 and H9N2 was dramatically decreased when incubated with **HSA-PEG2000-OA** conjugate, indicating its capture efficiency, For H3N2, the activity was less pronounced; we are not clear why this is the case at this time. It was also found that **HSA-PEG2000-OMe** has a moderate adsorption activity, which may be attributed to the nonspecific electrostatic interactions between the positive charges of cations on the viral surface and negative charges on the macromolecule surface[45]. Thus the virus would be captured and further form the conjugation complex, which will be easily precipitated by centrifugation. This study strongly demonstrated **HSA-PEG2000-OA** can capture the virus *via* specific adsorption to the HA on the virial surface. These results clearly indicate the OA modified neoprotein functions similarly to natural mucin.

Table 3. Capture of influenza virus by HSA-OA conjugate

Compounds	Virus	Relative NA activity
HSA-PEG2000-OMe	H1N1	49%
	H3N2	56%
	H9N2	48%
HSA-PEG2000-OA	H1N1	15%
	H3N2	43%
	H9N2	19%

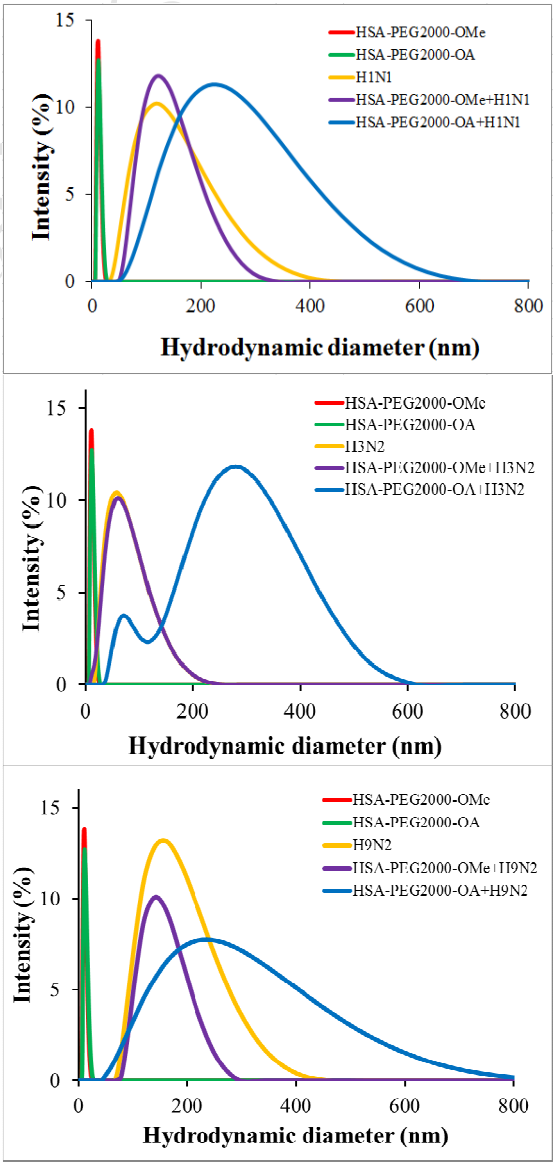


Fig 1. Hydrodynamic size distribution curves of the influenza virus a) Influenza A/Puerto Rico/8/1934 (H1N1), b) Influenza A/Huairou, Beijing/11069/2014 (H3N2), c) Influenza A/Chicken/Beijing/AT609/2014 (H9N2) before and after mixed with **HSA-PEG2000-OA** or **HSA-PEG2000-OMe**.

2.2.4. Isothermal Titration Calorimeter, ITC

To quantitatively analyze the interactions between virus and the HSA-OA conjugate, ITC was then performed to characterize the thermodynamic binding affinity[46,47]. The number of the virus was determined by haemagglutination titration which converted to virus particles by multiplying by 10^9. The concentration of virus particles was calculated on the basis of 10^5 particles/L equivalent 10^{-13} mol/L[48,49]. In each ITC experiment, the **HSA-PEG2000-OA** solution in PBS was loaded into the syringe and titrated into the influenza virus solution in the calorimeter cell. The heat released at each injection was used to calculate the thermodynamic parameters as described in the experimental section. **HSA-PEG2000-OMe** was used as the negative control (Table 4).

Table 4. Disassociation constant K_D of HSA conjugate–influenza virus interactions measured by ITC

Virus	**HSA-PEG2000-OMe**	**HSA-PEG2000-OA** (μM)
H1N1	NB[a]	32.6
H3N2	NB	20.9
H9N2	NB	21.4

[a] No binding

Significant binding of **HSA-PEG2000-OA** to all strains was observed with K_D in the μM range. A representative figure of the ITC graphs with influenza virus H9N2 (Figure 2a) depicts large favorable enthalpic and entropic contributions. In contract, no binding was detected for **HSA-PEG2000-OMe** (Figure 2b), indicating the specific binding was derived from the OA motif. To the best of our knowledge, this is the first time measuring the K_D value with intact virus from ITC experiments, which clearly highlighted the feasibility for the quantitative investigation of the interactions between biomacromolecules and intact viruses.

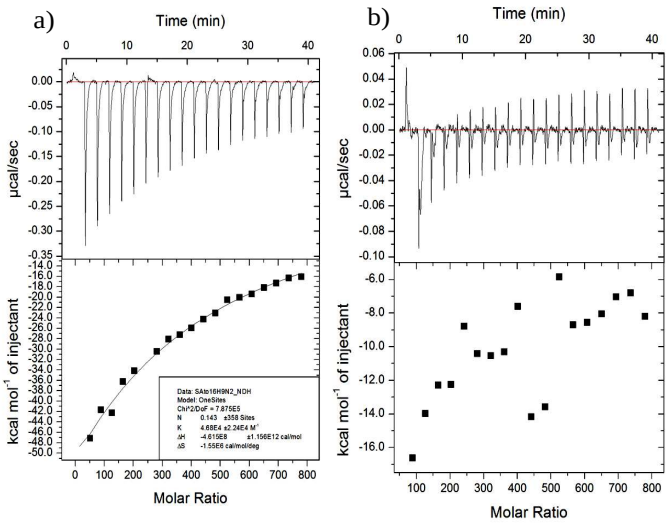


Fig 2. ITC titration curves obtained at 298 K for the titration of **HSA-PEG2000-OA** (60 μM) with a) 16 nM Influenza A/Chicken/Beijing/AT609/2014 (H9N2); b) 16 nM Influenza A/Chicken/Beijing/AT609/2014 (H9N2). The top panel showed the raw calorimetric data. Binding parameters were auto-generated after curve fitting using Microcal Origin 7.

2.2.5. Inhibition of H7N9 pseudovirus

Finally, a cell based assay was used to demonstrate the anti-infective properties of our lead compound. Briefly, a single-cycle H7N9 pseudovirus enveloped with influenza surface protein HA and NA, and packed with HIV backbone carrying luciferase reporter gene to simulate the actual infection process was used to evaluate the inhibitory activity of **HSA-PEG2000-OA** [50]. This assay system is an ideal platform for the virus entry inhibition study (Figure 3). The quantity of virus represented by luciferase activity, was determined by the luciferase reporter gene assay using fluorescence microplate reader for chemiluminescence following the protocols provided by the manufacture (Promega).

As shown in the insert column in Figure 3, unimolecular **OA** or **HSA-PEG2000-OMe** exhibited low inhibitory activity against H7N9 pseudovirus at 20 μM, probably due to the nonspecific electrostatic interactions with viral particles. In contrast, the **HSA-PEG2000-OA** inhibitory activity is much higher. These data suggested that our non sialyl protein conjugate could potentially be considered as a simpler alternative strategy for the construction of mucin mimic as antiviral barriers and entry inhibitor.

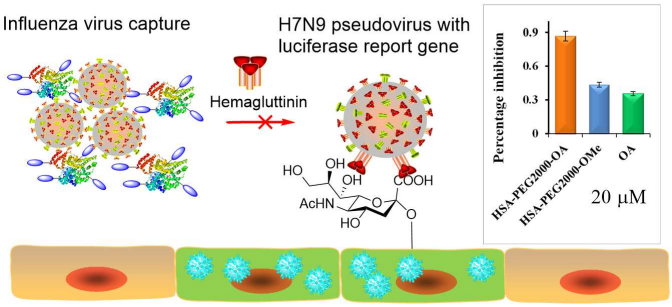


Fig 3. Schematic illustration of pseudovirus infection assay. Insert: inhibitory activity of **HSA-PEG2000-OA (**data derived from the mean of three independent experiments).

3. Conclusions

OA functionalized HSA was prepared via squaric acid diethyl esters strategy as nonglycan protein conjugates for mucin mimic. The multivalent binding to influenza virus was probed by HAI, DLS, ITC and virus capture assay. The antiviral activity was also investigated by in vitro infection assays. The results showed that this PEGylated OA-HSA conjugate exhibit strong virial capture capability and entry inhibitory activity. Our results compare well with other reports that use cyclodextrin-OA conjugates[25,26] for virus inhibition.

4. Experimental

4.1. Material

All materials were obtained from commercial suppliers, and were used without further purification. All solvents were commercially available grade. Thin-layer chromatography (TLC) was purchased from EMD Co. Ltd. (German). All compounds were stained with 5% phosphomolybdic acid in ethanol or iodine vapors. Spots of compounds were also visualized with UV light when possible. Flash column chromatography was performed on silica gel 200-300 mesh. The final OA analogs and protein conjugates were purified using SephadexTM LH-20 and SephadexTM G-75 (GE Healthcare), respectively. Human Serum Albumin was purchased from Sigma-Aldrich. Influenza viruses were obtained from National Institute for Viral Disease Control and Prevention, China CDC and propagated in 9 days old embryonated chicken eggs. The allantoic fluid was collected, then centrifuged at 3000 rpm min-1 for 10 min and the supernatant was stored at -80 ℃. Virus was inactivated by the addition of β -propiolactone (β -PL) when assays were conducted. H7N9 pseudoviruses was kindly provided by Dr. Hai-

Xia Xiao, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences.

4.2. Measurements

NMR spectra were recorded on Bruker AVANCE III (400 MHz) instruments. Chemical shifts (δ) were reported in parts per million downfield from TMS, the internal standard; *J* values were given in Hertz. The molecular weights of the OA conjugates were confirmed by Bruker ultrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Fluorescence and chemiluminescence intensity was measured using the Synergy™ H1/H1MF microplate reader (BioTek Instruments, Inc. USA). Dynamic light scattering was recorded on a Zata Sizer Nano apparatus (ZEN3690, Malvern Instruments Ltd, U.K.). Microcalorimetric experiments were performed with an Isothermal Titration Calorimeter (ITC200) (Microcal Inc., Northampton, MA). Solutions of the analytes in buffer were filtered through a 0.22 μm micro membrane and degassed before being injected.

4.3. Chemistry

4.3.1. General procedure for the esterification of oleanolic acid

To a solution of oleanolic acid (1.1 eq) and bromide (1 eq) in DMF K_2CO_3, (2 eq) was added. The reaction mixture was stirred at rt for 6–18 h and concentrated *in vacuo*. The residue was diluted with CH_2Cl_2, and the extract was washed successively with 1 M HCl, satd. $NaHCO_3$, and brine, dried with Na_2SO_4, filtered, and concentrated *in vacuo*. The residue was purified by column chromatography.

*4.3.2. 6-Azidohexyl 3β-hydroxyolean-12-en-28-oate (**C6-OA**, **2**)*

Prepared from **OA** (2.44 g, 5.34 mmol) and 1-azido-6-bromohexane (1 g, 4.85 mmol) according to the general procedure 4.3.1. The residue was purified by column chromatography [petroleum ether (PE)/EtOAc, 20/1 v/v] to give ***C6-OA***, ***2*** as colorless oil (1.18 g, 42%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.18 (t-like, 1H), 4.28 (d, *J* = 5.1 Hz, 1H), 3.94 (dd, *J* = 6.0, 4.4 Hz, 2H), 3.31 (d, *J* = 5.3 Hz, 2H), 3.02 – 2.97 (m, 1H), 2.79 (dd, *J* = 13.6, 3.9 Hz, 1H), 1.99 – 1.92 (m, 1H), 1.81 (d, *J* = 6.9 Hz, 2H), 1.63 – 1.40 (m, 16H), 1.34 – 1.30 (m, 5H), 1.24 – 1.20 (m, 2H), 1.10 (s, 4H), 0.89, 0.88, 0.85, 0.67, 0.66 (5s, 21H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 176.97, 143.93, 122.33, 79.67, 77.25, 64.00, 55.26, 51.03, 47.51, 46.48, 45.87, 41.69, 41.37, 38.83, 38.53, 37.02, 33.67, 33.21, 32.86, 32.58, 30.82, 28.74, 28.67, 28.46, 27.54, 27.42, 26.22, 26.05, 25.64, 23.76, 23.40, 23.02, 18.45, 17.16, 16.46, 15.48.

*4.3.3. 2-(2-(2-azidoethoxy) ethoxy) ethyl 3β-hydroxyolean-12-en-28-oate (**OEG-OA**, **3**)*

prepared from **OA** (2.1 g, 4.62 mmol) and 1-azido-2-(2-(2-bromoethoxy)ethoxy)ethane (1 g, 4.2 mmol) according to the general procedure 4.3.1. The residue was purified by column chromatography (PE/EtOAc, 15/1 v/v) to give ***OEG-OA***, ***3*** as colorless oil (1.15 g, 45%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.24 (t-like, *J* = 3.7 Hz, *J* = 3.3 Hz, 1H), 4.34 (d, *J* = 4.9 Hz, 1H), 4.19 – 4.06 (m, 2H), 3.67 – 3.64 (t, *J* = 4.8 Hz, 4H), 3.61 (s, 3H), 3.45 – 3.42 (m, 2H), 3.06 – 3.04 (m, 1H), 2.87 – 2.83 (m, 1H), 2.05 – 1.98 (m, 1H), 1.87 – 1.86 (m, 1H), 1.69 – 1.61 (m, 4H), 1.57 – 1.48 (m, 8H), 1.39 – 1.35 (m, 2H), 1.32 – 1.08 (m,6H), 0.95, 0.94, 0.91, 0.73 (4s, 21H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 177.02, 143.83, 122.37, 77.28, 70.33, 70.24, 69.82, 68.86, 63.71, 55.27, 50.49, 47.53, 46.49, 45.89, 41.70, 41.35, 38.85, 38.56, 37.04, 33.67, 33.22, 32.82, 32.47, 30.83, 29.52, 28.68, 27.57, 27.43, 26.07, 23.79, 23.41, 23.01, 18.45, 17.11, 16.48, 15.54.

*4.3.4. **OH-PEG2000-NH$_2$**, **8***

To a stirred solution of diol **HO-PEG2000-OH** (2g, 1 mmol) in CH_2Cl_2, fresh Ag_2O (350 mg, 1.5 mmol), TsCl (210 mg, 1.12 mmol) and KI (33 mg, 0.2 mmol) was added. The reaction mixture was stirred at rt for 4 h, filtered with celite and washed with CH_2Cl_2. The solvent was evaporated and the residue was directly dissolved in 25% ammonia and NH_4Cl (0.69 g, 12.94 mmol) was added. The reaction mixture was stirred at rt for 12 h, and extracted with CH_2Cl_2. The organic extracts were dried over Na_2SO_4. The solvent was removed under reduced pressure and the residue purified by gradient flash silica gel column chromatography to give H_2N-PEG-OH (1.4 g, 87.5%). The product was further purified by recrystallization from diethyl ether. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.84 – 3.82 (t, *J* = 4.8 Hz, 2H), 3.58 (br, 173H), 3.14 – 3.11 (t, *J* = 4.8 Hz, 2H).

*4.3.5. **Br-PEG2000-NHBoc**, **10***

TEA (211 mg, 2.07 mmol) was added to a stirred solution of **HO-PEG2000-NH$_2$** in CH_2Cl_2. After stirring for 0.5 h, $(Boc)_2O$ was slowly added to the reaction mixture, and further stirred at rt overnight. The reaction mixture was washed using 1 M HCl and extracted with CH_2Cl_2 three times, and the organic extracts were dried over Na_2SO_4. The solvent was removed under reduced pressure and the residue (**OH-PEG2000-NHBoc**, **9**) was dissolved in CH_2Cl_2/Et_3N and TsCl (3 eq.) was added at 0 ℃. The resulting solution was stirred for 8 h at rt. Upon completion, the reaction mixture was washed by HCl (1 M) and extracted with CH_2Cl_2 three times. The organic phase was combined, washed with brine, dried over Na_2SO_4 and evaporated to dryness. The residue was dissolved in dry acetone and lithium bromide (4 eq.) was added. The reaction mixture was stirred and refluxed for 12 h. The suspension was filtered and the filtrate was concentrated. The residue was washed with distilled water and extracted with CH_2Cl_2 three times. The organic phase was combined, washed with brine, dried over Na_2SO_4 and evaporated to dryness. The residue was purified by gradient flash silica gel column chromatography to give ***Br-PEG2000-NHBoc***, **10**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.06 (s, 1H), 3.74 – 3.23 (m, 213 H), 1.37 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.93, 71.13, 70.50, 70.13, 40.29, 30.33, 28.39.

*4.3.6. **PEG2000-OA**, **4***

Prepared from **OA** (68 mg, 0.15 mmol) and **Br-PEG2000-NHBoc** (0.3 g, 0.14 mmol) according to the general procedure 4.3.1. The residue was purified by column chromatography (CH_2Cl_2/MeOH, 20/1 v/v) to yield **4** as colorless oil(340 mg, 97%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.21 (s, 1H), 5.06 (s, 1H), 4.15 – 4.06 (m, 2H), 3.75 (s, 2H), 3.58 (s, 188H), 3.48 – 3.46 (m, 3H), 3.40 (d, *J* = 4.4 Hz, 2H), 3.24 (d, *J* = 4.5 Hz, 2H), 3.14 – 3.12 (d, *J* = 6.7 Hz, 1H), 2.84 – 2.78 (m, 1H), 2.50 (s, 2H), 1.93 – 1.80 (m, 2H), 1.62 – 1.46 (m, 9H), 1.37 (s, 9H), 1.30 – 1.19 (m, 4H), 1.06, 0.91, 0.85, 0.83, 0.71, 0.66 (6s, 21H).^{13}C NMR (100 MHz, $CDCl_3$) δ 177.54, 155.97, 143.67, 122.36, 78.79, 70.53, 70.18, 69.12, 63.31, 55.19, 47.57, 46.62, 45.84, 41.64, 41.24, 40.32, 39.28, 38.71, 38.42, 36.99, 33.83, 33.09, 32.69, 32.33, 30.66, 28.41, 28.11, 27.61, 27.16, 25.85, 23.60, 23.38, 22.94, 18.30, 16.96, 15.61, 15.31.

4.3.7. General procedure for the squaric acid monoamination

A solution of azide in methanol and CH_2Cl_2 (1/1 v/v) was hydrogenated with Lindlar catalyst under an atmosphere of hydrogen for 12 h. Then the suspension was filtered through a pad of Celite, and washed with methanol. The filtrate was concentrated under reduced pressure to give colorless foam, which was dissolved in carbonate buffer (7.0 mL, pH 7.0). Diethyl squarate (2 eq, based on azide) was added, and the solution was slowly stirred at rt for 14 h. The solvent was removed *in vacuo* and the residue was purified by flash column chromatography.

*4.3.8. 6-(2-ethoxy-3, 4-dioxocyclobut-1-en-1-amino) hexyl 3β-hydroxyolean-12-en-28-oate (**5**)*

Prepared from **2** (1.18 g, 2.03 mmol) according to the general procedure 4.3.7. The residue was purified by column chromatography (PE/EtOAc, 2/1 v/v) to give **5** as colorless syrup (560 mg, 50%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.18 (t-like,

1H), 4.69 – 4.64 (m, 2H), 4.32 (d, *J* = 5.0 Hz, 1H), 3.95 (t, *J* = 5.6 Hz, 2H), 3.48 – 3.45 (m, 1H), 3.30 – 3.25 (m, 2H), 3.01 – 2.97 (m, 1H), 2.80 (d, *J* = 10.0 Hz, 1H), 2.00 – 1.92 (m, 1H), 1.81 (s, 2H), 1.67 – 1.43 (m, 16H), 1.39 – 1.36 (m, 5H), 1.34 – 1.31 (m, 6H), 1.10 (s, 4H), 0.90, 0.89, 0.83, 0.67, 0.66 (5s, 21H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 189.73, 182.47, 176.94, 143.91, 122.34, 79.66, 77.28, 69.13, 64.01, 55.29, 47.52, 46.47, 45.89, 44.20, 43.87, 41.69, 41.38, 39.35, 38.82, 38.55, 37.01, 33.69, 33.21, 32.88, 32.58, 30.80, 30.40, 28.66, 28.44, 27.55, 27.41, 26.04, 25.89, 25.62, 23.76, 23.40, 23.04, 18.45, 17.16, 16.44, 16.10, 15.47.

*4.3.9. 2-[2-(2-2-ethoxy-3, 4-dioxocyclobut-1-en-1-amino) ethoxy] ethoxy ethyl 3β-hydroxyolean-12-en-28-oate (**6**)*

Prepared from **3** (1.15 g, 1.87 mmol) according to the general procedure 4.3.7. The residue was purified by column chromatography (PE/EtOAc, 2/1 v/v) to give **6** as white syrup (350 mg, 36%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.23 (t-like, *J* = 3.7 Hz, *J* = 3.3 Hz, 1H), 4.71 (q, *J* = 6.7 Hz, 2H), 4.37 (d, *J* = 5.0 Hz, 1H), 4.18 – 4.04 (m, 2H), 3.68 – 3.66 (m, 1H), 3.63 – 3.62 (t, *J* = 4.1 Hz, 2H), 3.58 (s, 6H), 3.49 – 3.48 (m, 1H), 3.06 – 3.02 (m, 1H), 2.85 – 2.81 (m, 1H), 2.03 – 1.97 (m, 1H), 1.86 – 1.85 (m, 1H), 1.68 – 1.60 (m, 4H), 1.56 – 1.50 (m, 8H), 1.46 – 1.43 (m, 5H), 1.14 (s, 4H), 0.95, 0.93, 0.90, 0.72, 0.71 (5s, 21H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 189.71, 189.56, 182.67, 182.53, 176.93, 143.77, 122.37, 79.64, 77.30, 70.31, 70.20, 69.77, 69.18, 68.86, 63.62, 55.30, 47.55, 46.47, 45.89, 44.18, 41.68, 41.34, 40.65, 40.44, 40.23, 40.02, 39.82, 39.61, 39.40, 38.84, 38.58, 37.03, 33.71, 33.23, 32.84, 32.46, 30.80, 28.66, 27.57, 27.42, 26.06, 23.77, 23.41, 23.01, 18.46, 17.09, 16.44, 16.11, 15.54.

*4.3.10. **PEG2000-OA**-amino squaric acid monoethyl ester, (**7**)*

To a solution of ***PEG2000-OA***, ***4*** in CH_2Cl_2, trifluoroacetic acid /CH_2Cl_2 (1/1 v/v) was added under 0 ℃, and the reaction mixture was stirred for 20 min at room temperature. The solution was evaporated to dryness. The crude material was directly used in next step. **PEG2000-OA**-monoethyl ester was prepared according to the general procedure 4.3.7. The residue was purified by column chromatography (CH_2Cl_2/MeOH, 15/1 v/v) to give **7** as white syrup (180 mg, 63%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.21 (t-like, 1H), 4.69 (q-like, *J* = 6.7 Hz, 2H), 4.16 (t, *J* =4.5 Hz, 2H), 3.58 (s, 187H), 3.40 (t, *J* =5.2 Hz, 2H), 3.17 – 3.13 (m, 1H), 2.80 (d, *J* = 9.6 Hz, 1H), 2.67 (d, *J* = 11.7 Hz, 1H), 2.42 (s, 4H), 2.12 (d, *J* = 6.8 Hz, 1H), 1.90 – 1.80 (m, 3H), 1.70 – 1.53 (m, 8H), 1.48 – 1.44 (m, 3H), 1.42 – 1.37 (m, 4H), 1.18 (s, 3H), 1.07 (d, *J* = 8.6 Hz, 4H), 0.91, 0.85, 0.83, 0.80, 0.71, 0.69, 0.66 (7s, 21H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.60, 143.78, 137.88, 128.10, 122.09, 86.28, 70.54, 69.43, 69.12, 63.32, 63.19, 55.25, 50.59, 48.44, 47.50, 46.62, 45.83, 44.29, 41.66, 41.31, 39.30, 38.32, 38.00, 37.93, 37.19, 36.87, 36.80, 35.79, 34.78, 33.84, 33.08, 32.93, 32.65, 32.16, 30.67, 29.65, 27.86, 26.98, 25.82, 25.07, 24.13, 23.60, 23.19, 21.63, 20.96, 18.13, 17.50, 16.94, 16.35, 15.34.

*4.3.11. CH_3O-PEG- amino squaric acid monoethyl ester, (**13**)*

Prepared from **12** (2.3 g, 1.13 mmol) according to the general procedure 4.3.7. The residue was recrystallized in ethyl ether to give (1.40 g, 82%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.76 (q-like, *J* = 6.9 Hz, 2H), 3.64 (s, 185H), 3.38 (s, 3H), 1.46 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 71.89, 70.53, 69.40, 58.98, 15.84.

4.3.12. General procedure for the modification of HSA with OA monoethyl ester

HSA (1 eq) was dissolved in carbonate buffer (pH 9.0). When all protein had dissolved, OA monoethyl ester (20 eq) was added, and the soln was slowly stirred at rt for 18 h. The reaction mixture was transferred to an Amicon ultrafiltration device (MWCO 10 kDa, Millipore) and desalted by repeatedly adding deionized water to the upper chamber. The water was evaporated to 2 mL and purified by Sephadex® G-75 using deionized water as eluent then lyophilisation. The characterization of the conjugate was performed using MALDI-TOF-MS in positive linear ion mode using 2, 5-dihydroxybenzoic acid (DHB) as matrix. FlexAnalysis was used for analysis of the data.

4.4. Bioevaluation

4.4.1. Hemagglutination inhibition (HAI) assay

HAI assays were performed according to the method described previously[29].

4.4.2. Binding Assay

4.4.2.1. Dynamic Light Scattering (DLS)

The hydrodynamic diameters of the **HSA-PEG2000-OA** with or without the influenza virus were measured by dynamic light scattering. **HSA-PEG2000-OA** or virus in phosphate buffered saline (pH 7.4) was individually filtered through 0.22 μm water membrane filters. Glycoconjugates and different strains of influenza virus were mixed. The resulted solution without further filtration was transferred into a sample cell for measurement. DLS measurement was done on a Zata Sizer Nano apparatus (ZEN3690, Malvern Instruments Ltd, U.K.). Data analysis was performed using the software provided with the instrument.

4.4.2.2. Virus Capture Assay

The resulted mixtures of **HSA-PEG2000-OA/ HSA-PEG2000-OMe** and different strains of influenza virus in 4.2.2.1 were centrifuged (3000 rpm, 5 min), respectively. The supernatant was transferred, and the quantity of virus was represented by the NA activity with 4-methylumbelliferyl-a-*D*-N-acetylneuraminic acid sodium salt (4-MUNANA) as the fluorescent substrate. The solution in the absence of conjugates with virus was used as control.

4.4.2.3. Isothermal titration calorimetry (ITC)

ITC experiments were performed using an ITC200 Microcalorimeter in PBS buffer. The number of the viral particles was determined by haemagglutination titration which converted to virus particles by multiplying 10^9 as described previously[48,49]. The concentration of virus particles was calculated on the basis of 10^5 particles/L equivalent to 10^{-13} mol/L. The concentration of HSA conjugates was 60 μM, and that of influenza virus H1N1, H3N2, H9N2 was 4, 8 and 16 nM, respectively to get a good fit. In each individual experiment, ~38 μL of 40 μM conjugate solution was injected through the computer-controlled 40 μL microsyringe at an interval of 2 min into the viral solution in the same buffer (cell volume = 200 μL) while stirring at 750 rpm. The experimental data were fitted to a theoretical titration curve using the software supplied by MicroCal. A standard one-site model was used with ΔH (enthalpy change, in kcal/mol), K (association constant, in M^{-1}), and N (number of binding sites) as the variables.

4.4.3. Entry inhibitory activity against H7N9 pseudovirus

MDCK cells (10^4/well) were seeded in 96-well plates and grown overnight. H7N9 pseudotyped particles [HA plasmid from the H7 subtype strain A, NA plasmid from the N1 subtype strain, HIV backbone plasmid (pNL4-3.luc.R_E)] was incubated with appropriate concentrations of tested OA derivates for 30 min at 37 ℃. Subsequently, the virus-OA derivate mixture was transferred to the cells and incubated for an additional 48 h. Cells were washed with phosphate buffer saline (PBS) and lysed with luciferase cell culture lysis reagent (Promega, Madison, WI). Aliquots of cell lysates were transferred to 96-well flat bottom luminometer plates (Costar), followed by the addition of luciferase assay substrate (Promega). The luciferase activity was measured in a microplate reader (BioTek Instruments, Inc. USA). As a negative control, H7N9 pseudotyped particles were incubated with **HSA-PEG2000-OMe**. The yield of pseudoviruses in MDCK cells were reflected by luminescence intensity.

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References and notes

(1) National Health and Family Planning Commission of the People's Republic of China, Reports of Nationally Notifiable Infectious Diseases, Available online: http://www.nhfpc.gov.cn/jkj/s3578/201702/f1e4cfe184e44f80ae57d0954c3d5fce.shtml, Accessed Auguest 25, 2017.

(2) National Health and Family Planning Commission of the People's Republic of China, Reports of Nationally Notifiable Infectious Diseases, Available online: http://www.nhfpc.gov.cn/jkj/s3578/new_list.shtml, Accessed Auguest 25, 2017.

(3) von Itzstein, M.; Wu, W. Y.; Kok, G. B.; Pegg, M. S.; Dyason, J. C.; Jin, B.; Van Phan, T.; Smythe, M. L.; White, H. F.; Oliver, S. W.; et al. Rational design of potent sialidase-based inhibitors of influenza virus replication. *Nature* **1993**, *363*, 418-423.

(4) Kim, C. U.; Lew, W.; Williams, M. A.; Liu, H.; Zhang, L.; Swaminathan, S.; Bischofberger, N.; Chen, M. S.; Mendel, D. B.; Tai, C. Y.; Laver, W. G.; Stevens, R. C. Influenza neuraminidase inhibitors possessing a novel hydrophobic interaction in the enzyme active site: design, synthesis, and structural analysis of carbocyclic sialic acid analogues with potent anti-influenza activity. *J. Am. Chem. Soc.* **1997**, *119*, 681-690.

(5) Danqi, B.; Li, Z.; Liu, Q.; Richt, J. A. H7N9 avian influenza A virus in China: a short report on its circulation, drug resistant mutants and novel antiviral drugs. *Expert Rev. Anti Infect. Ther.* **2017**, 1-5.

(6) Chinese Center for Disease Control and Prevention. Human infection with avian influenza A(H7N9) virus – China. Available from:http://www.chinacdc.cn/jkzt/crb/zl/rgrgzbxqlgg/rgrqlgyp/201702/t20170221_138523.html.

(7) National Health and Family Planning Commission of the People's Republic of China, Notification of medical treatment for human influenza H7N9 infection, Available online: http://www.nhfpc.gov.cn/yzygj/s3593g/201401/3f69fe196ecb4cfc8a2d6d96182f8b22.shtml.

(8) De Clercq, E. Antiviral agents active against influenza A viruses. *Nat. Rev. Drug. Discov.* **2006**, *5*, 1015-1025.

(9) Das, K.; Aramini, J. M.; Ma, L. C.; Krug, R. M.; Arnold, E. Structures of influenza A proteins and insights into antiviral drug targets. *Nat. Struct. Mol. Biol.* **2010**, *17*, 530.

(10) Loregian, A.; Mercorelli, B.; Nannetti, G.; Compagnin, C.; Palu, G. Antiviral strategies against influenza virus: towards new therapeutic approaches. *Cell Mol. Life Sci.* **2014**, *71*, 3659-3683.

(11) Linden, S. K.; Sutton, P.; Karlsson, N. G.; Korolik, V.; McGuckin, M. A. Mucins in the mucosal barrier to infection. *Mucosal Immunol.* **2008**, *1*, 183-197.

(12) Lieleg, O.; Lieleg, C.; Bloom, J.; Buck, C. B.; Ribbeck, K. Mucin biopolymers as broad-spectrum antiviral agents. *Biomacromolecules* **2012**, *13*, 1724-1732.

(13) Skehel, J. J.; Wiley, D. C. Receptor Binding and Membrane Fusion in Virus Entry: The Influenza Hemagglutinin. *Annu. Rev. Biochem.* **2000**, *69*, 531-569.

(14) Palese, P.; Tobita, K.; Ueda, M.; Compans, R. W. Characterization of temperature sensitive influenza virus mutants defective in neuraminidase. *Virology* **1974**, *61*, 397-410.

(15) Bhatia, S.; Camacho, L. C.; Haag, R. Pathogen Inhibition by Multivalent Ligand Architectures. *J. Am. Chem. Soc.* **2016**, *138*, 8654-8666.

(16) Sun, X. L. Recent anti-influenza strategies in multivalent sialyloligosaccharides and sialylmimetics approaches. *Curr. Med. Chem.* **2007**, *14*, 2304-2313.

(17) Wilson, J. C.; Kiefel, M. J.; Angus, D. I.; von Itzstein, M. Investigation of the Stability of Thiosialosides toward Hydrolysis by Sialidases Using NMR Spectroscopy. *Org. Lett.* **1999**, *1*, 443-446.

(18) He, Y.; Yang, Y.; Iyer, S. S. Neuraminidase Resistant Sialosides for the Detection of Influenza Viruses. *Bioconjug. Chem.* **2016**, *27*, 1509-1517.

(19) Yeh, H.-W.; Lin, T.-S.; Wang, H.-W.; Cheng, H.-W.; Liu, D.-Z.; Liang, P.-H. S-Linked sialyloligosaccharides bearing liposomes and micelles as influenza virus inhibitors. *Org. & Biomol. Chem.* **2015**, *13*, 11518-11528.

(20) Kale, R. R.; Mukundan, H.; Price, D. N.; Harris, J. F.; Lewallen, D. M.; Swanson, B. I.; Schmidt, J. G.; Iyer, S. S. Detection of intact influenza viruses using biotinylated biantennary S-sialosides. *J. Am. Chem. Soc.* **2008**, *130*, 8169-8171.

(21) Li, X.; Wu, P.; Gao, G. F.; Cheng, S. Carbohydrate-functionalized chitosan fiber for influenza virus capture. *Biomacromolecules* **2011**, *12*, 3962-3969.

(22) Ogata, M.; Murata, T.; Murakami, K.; Suzuki, T.; Hidari, K. I. P. J.; Suzuki, Y.; Usui, T. Chemoenzymatic synthesis of artificial glycopolypeptides containing multivalent sialyloligosaccharides with a γ-polyglutamic acid backbone and their effect on inhibition of infection by influenza viruses. *Bioorg. Med. Chem.* **2007**, *15*, 1383-1393.

(23) Totani, K.; Kubota, T.; Kuroda, T.; Murata, T.; Hidari, K. I. P. J.; Suzuki, T.; Suzuki, Y.; Kobayashi, K.; Ashida, H.; Yamamoto, K.; Usui, T. Chemoenzymatic synthesis and application of glycopolymers containing multivalent sialyloligosaccharides with a poly(L-glutamic acid) backbone for inhibition of infection by influenza viruses. *Glycobiology* **2003**, *13*, 315-326.

(24) Yu, M.; Si, L.; Wang, Y.; Wu, Y.; Yu, F.; Jiao, P.; Shi, Y.; Wang, H.; Xiao, S.; Fu, G.; Tian, K.; Wang, Y.; Guo, Z.; Ye, X.; Zhang, L.; Zhou, D. Discovery of pentacyclic triterpenoids as potential entry inhibitors of influenza viruses. *J. Med. Chem.* **2014**, *57*, 10058-10071.

(25) Tian, Z.; Si, L.; Meng, K.; Zhou, X.; Zhang, Y.; Zhou, D.; Xiao, S. Inhibition of influenza virus infection by multivalent pentacyclic triterpene-functionalized per-O-methylated cyclodextrin conjugates. *Eur. J. Med. Chem.* **2017**, *134*, 133-139.

(26) Xiao, S.; Si, L.; Tian, Z.; Jiao, P.; Fan, Z.; Meng, K.; Zhou, X.; Wang, H.; Xu, R.; Han, X.; Fu, G.; Zhang, Y.; Zhang, L.; Zhou, D. Pentacyclic triterpenes grafted on CD cores to interfere with influenza virus entry: A dramatic multivalent effect. *Biomaterials* **2016**, *78*, 74-85.

(27) Cecioni, S.; Imberty, A.; Vidal, S. Glycomimetics versus Multivalent Glycoconjugates for the Design of High Affinity Lectin Ligands. *Chem. Rev.* **2015**, *115*, 525-561.

(28) Lundquist, J. J.; Toone, E. J. The Cluster Glycoside Effect. *Chem. Rev.* **2002**, *102*, 555-578.

(29) Yang, Y.; Liu, H. P.; Yu, Q.; Yang, M. B.; Wang, D. M.; Jia, T. W.; He, H. J.; He, Y.; Xiao, H. X.; Iyer, S. S.; Fan, Z. C.; Meng, X.; Yu, P. Multivalent S-sialoside protein conjugates block influenza hemagglutinin and neuraminidase. *Carbohydrate research* **2016**, *435*, 68-75.

(30) Wang, D.-M.; Meng, X.; Li, X.-B.; He, H.-J.; Zhao, T.-F.; Jia, T.-W.; He, Y.; Yang, Y.; Yu, P.: Modification of bovine serum albumin with aminophenylboronic acid as glycan sensor based on surface plasmon resonance and isothermal titration calorimetry. *Heterocycl. Commun.*, **2017**, *23*, 237-243.

(31) Cheng, K.; Liu, J.; Liu, X.; Li, H.; Sun, H.; Xie, J. Synthesis of glucoconjugates of oleanolic acid as inhibitors of glycogen phosphorylase. *Carbohydrate research* **2009**, *344*, 841-850.

(32) Tu, H. Y.; Huang, A. M.; Wei, B. L.; Gan, K. H.; Hour, T. C.; Yang, S. C.; Pu, Y. S.; Lin, C. N. Ursolic acid derivatives induce cell cycle arrest and apoptosis in NTUB1 cells associated with reactive oxygen species. *Bioorg. Med. Chem.* **2009**, *17*, 7265-7274.

(33) Tao, S.; Jia, T. W.; Yang, Y.; Chu, L. Q. BSA-Sugar Conjugates as Ideal Building Blocks for SPR-Based Glycan Biosensors. *ACS Sens.* **2017**, *2*, 57-60.
(34) Caliceti, P.; Veronese, F. M. Pharmacokinetic and biodistribution properties of poly(ethylene glycol)-protein conjugates. *Adv. Drug. Deliv. Rev.* **2003**, *55*, 1261-1277.
(35) Delgado, C.; Francis, G. E.; Fisher, D. The uses and properties of PEG-linked proteins. *Crit. Rev. Ther. Drug. Carrier Syst.* **1992**, *9*, 249-304.
(36) Li, J.; Crasto, C. F.; Weinberg, J. S.; Amiji, M.; Shenoy, D.; Sridhar, S.; Bubley, G. J.; Jones, G. B. An approach to heterobifunctional poly(ethyleneglycol) bioconjugates. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 5558-5561.
(37) Yang, Y.; He, Y.; Li, X.; Dinh, H.; Iyer, S. S. Bifunctional thiosialosides inhibit influenza virus. *Bioorg. Med. Chem. Lett.* **2014**, *24*, 636-643.
(38) Katz, J. M.; Hancock, K.; Xu, X. Serologic assays for influenza surveillance, diagnosis and vaccine evaluation. *Expert Rev. Anti. Infect. Ther.* **2011**, *9*, 669-683.
(39) Mammen, M.; Dahmann, G.; Whitesides, G. M. Effective Inhibitors of Hemagglutination by Influenza Virus Synthesized from Polymers Having Active Ester Groups. Insight into Mechanism of Inhibition. *J. Med. Chem.* **1995**, *38*, 4179-4190.
(40) Tang, S.; Puryear, W. B.; Seifried, B. M.; Dong, X.; Runstadler, J. A.; Ribbeck, K.; Olsen, B. D. Antiviral Agents from Multivalent Presentation of Sialyl Oligosaccharides on Brush Polymers. *ACS Macro. Letters* **2016**, *5*, 413-418.
(41) Driskell, J. D.; Jones, C. A.; Tompkins, S. M.; Tripp, R. A. One-step assay for detecting influenza virus using dynamic light scattering and gold nanoparticles. *The Analyst* **2011**, *136*, 3083-3090.
(42) Poonthiyil, V.; Nagesh, P. T.; Husain, M.; Golovko, V. B.; Fairbanks, A. J. Gold Nanoparticles Decorated with Sialic Acid Terminated Bi-antennary N-Glycans for the Detection of Influenza Virus at Nanomolar Concentrations. *ChemistryOpen* **2015**, *4*, 708-716.
(43) Barrett, S.; Mohr, P. G.; Schmidt, P. M.; McKimm-Breschkin, J. L. Real Time Enzyme Inhibition Assays Provide Insights into Differences in Binding of Neuraminidase Inhibitors to Wild Type and Mutant Influenza Viruses. *PLoS ONE* **2011**, *6*, e23627.
(44) Yu, M.; Wang, Y.; Tian, L.; Wang, Y.; Wang, X.; Liang, W.; Yang, J.; Yu, D.; Ma, T.; Fang, X. Safflomin A inhibits neuraminidase activity and influenza virus replication. *RSC Adv.* **2015**, *5*, 94053-94066.
(45) Ichiyama, K.; Yang, C.; Chandrasekaran, L.; Liu, S.; Rong, L.; Zhao, Y.; Gao, S.; Lee, A.; Ohba, K.; Suzuki, Y. Cooperative Orthogonal Macromolecular Assemblies with Broad Spectrum Antiviral Activity, High Selectivity, and Resistance Mitigation. *Macromolecules* **2016**.
(46) Freyer, M. W.; Lewis, E. A.: Isothermal titration calorimetry: Experimental design, data analysis, and probing Macromolecule/Ligand binding and kinetic interactions. In *Biophysical Tools for Biologists: Vol 1 in Vitro Techniques*; Correia, J. J., Detrich, H. W., Eds.; Methods in Cell Biology, **2008**, *84*, 79-113.
(47) Wang, X.; Matei, E.; Gronenborn, A. M.; Ramstrom, O.; Yan, M. Direct Measurement of Glyconanoparticles and Lectin Interactions by Isothermal Titration Calorimetry. *Anal. Chem.* **2012**, *84*, 4248-4252.
(48) Humphrey, A. J.; Fremann, C.; Critchley, P.; Malykh, Y.; Schauer, R.; Bugg, T. D. H. Biological Properties of N-Acyl and N-Haloacetyl Neuraminic Acids: Processing by Enzymes of Sialic Acid Metabolism, and Interaction with Influenza Virus. *Bioorg. Med. Chem.* **2002**, *10*, 3175-3185.
(49) Hidari, K. I. P. J.; Shimada, S.; Suzuki, Y.; Suzuki, T. Binding kinetics of influenza viruses to sialic acid-containing carbohydrates. *Glycoconj. J.* **2007**, *24*, 583-590.
(50) Song, G.; Shen, X.; Li, S.; Li, Y.; Si, H.; Fan, J.; Li, J.; Gao, E.; Liu, S. Structure-activity relationships of 3-O-beta-chacotriosyl oleanane-type triterpenoids as potential H5N1 entry inhibitors. *Eur. J. Med. Chem.* **2016**, *119*, 109-121.

Supplementary Material

^{1}H and ^{13}C NMR spectra of key intermediates and final glycopolymers and ITC raw data associated with this article can be found in the online version, at

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¶Oleanolic acid–human serum albumin conjugates as nonglycosylated neomucin mimic were prepared.

¶ITC technology was used in quantificational analysis of the conjugate – intact influenza affinity.

¶The conjugate can be used as influenza virus adsorbent and entry inhibitor.