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Synthesis of *S*-linked NeuAc-α(2-6)-di-LacNAc bearing liposomes for H1N1 influenza virus inhibition assays

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ABSTRACT

S-NeuAc-α(2-6)-di-LacNAc (**5**) was efficiently synthesized by a [2+2] followed by a [1+4] glycosylation, and later conjugated with 1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine (DLPE) to form both single-layer and multi-layer homogeneous liposomes in the presence of dipalmitoyl phosphatidylcholine (DPPC) and cholesterol. These liposomes were found to be weak inhibitors in both the influenza virus entry assay and the hemagglutination inhibition assay. The single layer liposome was found to more efficiently interfere with the entry of the H1N1 influenza virus into MDCK cells than the multilayer liposome containing **5**.

1. Introduction

Influenza is an infectious disease caused by the influenza virus, which infects humans and animals through contact with bronchial epithelial cells. The mechanism by which the virus attacks the cell entails recognition and cleavage of the terminal alpha-linked sialic acid on the surface of the cell by the glycoprotein hemagglutinin (HA), present on the surface of the virus. There are several different types of alpha-sialic acid linkage present on the surfaces of human mucosal cells, for which different types of influenza viruses have different binding preferences. For example, avian influenza virus binds to sialic acid in a α2-3-linkage with galactose; swine influenza virus is reported to bind sialic acid in either a α2-6 or α2-3 linkage with galactose; and human-adapted influenza viruses have binding preferences for a α2-6 sialic acid linkage.[1–3]

Following invasion of the cell in this way, a second influenza surface glycoprotein, neuraminidase (NA), is implicated in the release of the virus from the host cell. As both processes are integral to the proliferation of the virus, both HA and NA are promising targets for the development of anti-influenza drugs. NA inhibitors, such as oseltamivir and zanamivir, have seen clinical use for a decade.[4] Oseltamivir resistance,[5,6] however, is a growing problem; influenza continues to threaten humans; and new drugs to combat it are urgently required. Meanwhile, anti-influenza inhibitors that target HA are underdeveloped, compared to NA inhibitors.[7,8]

Although the targeting of HA is an option for the treatment of influenza, the binding between multiple HA of an influenza virus and the sialic acid surface receptors of an erythrocyte during viral infection is estimated to occur with an affinity of 10^{13} M^{-1}, whereas the association constant of a single sialic acid-HA interaction is 10^{3} M^{-1};[9] and thus the high degree of multi-valency required for a strong binding affinity between HA and HA-bound molecules poses a challenge.[10] For example, the sialic acid moieties presented by human mucosal cells are densely packed together, and any platform presenting an array of molecules intended to interact with the HA trimer would also have to be very densely packed.

When covalently displayed on the surface of liposome, *S*-Neu5Ac-α(2-6)-LacNAc (**2**, Fig. 1) was shown to have an EC_{50} of around 70–180 μM in a virus entry inhibitory assay.[11] Since the potency was not optimized, we sought to increase the inhibitory effect of sialic conjugation. A model to describe the binding between HA and the sialoside was recently disclosed.[12] When HA binds to α2-3-sialoside, it is believed to assume a "cone-like" topology. However, when HA binds to α2-6-sialoside, it forms an "umbrella-like" topology with additional glycan conformational flexibility. Longer α2-6 sialic acid bearing oligosaccharides would

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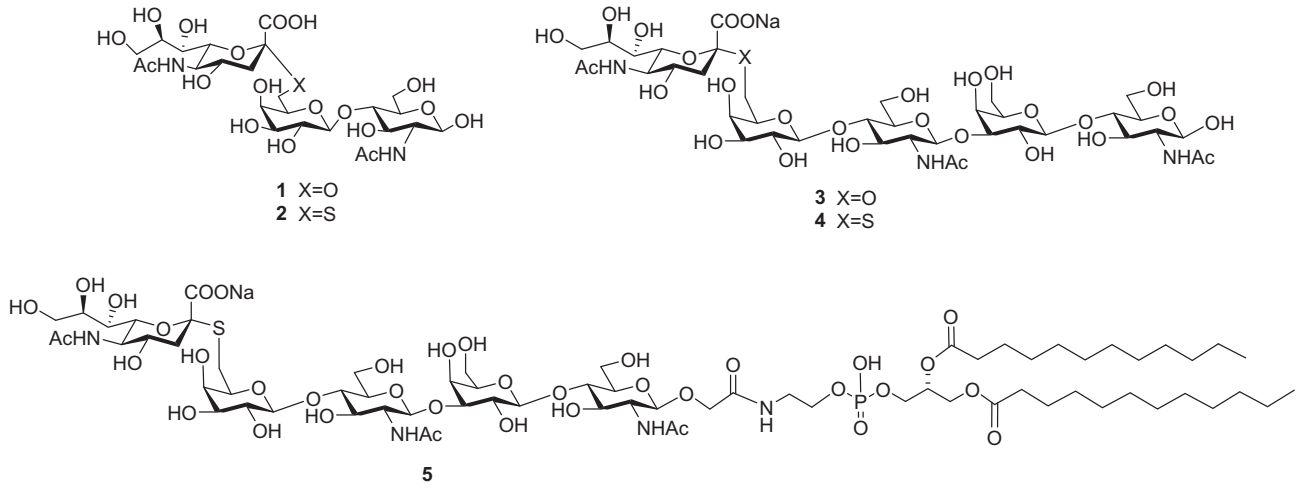

Fig. 1. Structure of sialic acid containing trisaccharides (**1**, **2**), pentasaccharides (**3**, **4**), and DLPE conjugation **5**.

favor this conformation. In mammalian respiratory epithelial cells, LacNAc is frequently displayed.[12,13] Glycan microarray studies revealed that the HAs of human influenzas bound best to *N*-glycans with the linear di-LacNAc sequence, and also to *N*-glycans with the tri-LacNAc repeat sequence.[14] Neu5Ac-α(2-6)-di-LacNAc (**3**) was indeed found to have a stronger binding preference for influenza than trisaccharide **1** (Fig. 1). Accordingly, we designed and synthesized *S*-Neu5Ac-α(2-6)-di-LacNAc (**4**), wherein the aforementioned trisaccharide **2** has been extended by LacNAc and *S*-linked Neu5Ac moieties.[15,16] This compound is expected to serve as both a novel synthetic recognition molecule of HA, and as a competitor of natural *O*-glycosides to NA.

Several multivalent vectors (dendrimers,[17] polymers,[18] liposomes,[19] and nanoparticles[20]), have been functionalized with sialic acid; and their abilities to inhibit the activities of their corresponding lectins were measured. However, in all cases, either heterogeneous mixtures of Neu5Ac were submitted for conjugation, and/or they contained only a Neu5Ac moiety for protein recognition, which may be not sufficient for HA interaction, and cannot be used to quantify the contribution of Neu5Ac in the virus interaction.[14] In order to procure a multivalent display of homogenous **4**, it was designed to conjugate with phospholipid – DLPE (1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine) to give **5** (Fig. 1), which could be formulated with DPPC (dipalmitoyl phosphatidylcholine) to form liposomes (Fig. 2) in aqueous solution. These compounds and formulations were evaluated in the A/WSN/33 H1N1 influenza entry inhibition and hemagglutination inhibition assays by red blood cells (RBC)s.

2. Results and discussion

2.1. Synthesis of DLPE bearing *5*

Our retrosynthesis of **5** is depicted in Scheme 1. The key steps are the formation of the amide bond that connects the lipid with the pentasaccharide; and two key glycosylation steps. Firstly, the *S*-glycosidic α2-6 linkage, would be formed by nucleophilic substitution of the triflate group at the galactose 6-position of compound **8** by the Neu5Ac thiolate of **7** ([1+4] glycosylation step); and secondly, the [2+2] glycosylation in step c, which would yield compound **8**. In order to control the selectivity of the glycosylations of steps c, d and e, benzoyl (Bz) and trichloroethylcarbonyl (Troc) groups would be introduced at the galactose 2-OH (compounds **11** and **14**) and glucosamine 2-NH_2 positions (compounds **12** and **13**), respectively. These are expected to confer a degree of β-selectivity on the glycosylation, by a way of neighboring-group partici-

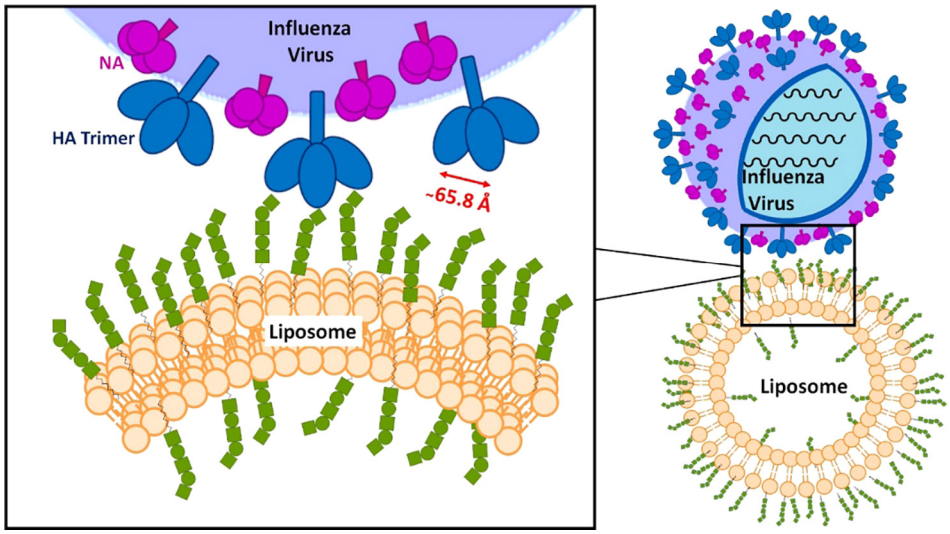

Fig. 2. Approach to present NA *S*-linked NeuAc-α(2-6)-di-LacNAc bearing liposomes to HA of influenza virus.