Construction of Effective Receptor for Recognition of Avian Influenza H5N1 Protein HA1 by Assembly of Monohead Glycolipids on Polydiacetylene Vesicle Surface

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Fast and sensitive detection of epidemic virus is of the utmost importance for human being in nowadays. Various biosensors have been designed for this goal based on conjugation event between host cell glycolipids and invading virus. However, multihead glycolipids analogous to native receptors on cell surface are known to be very difficult to mimic because of the complexity of chemical synthesis. Here, we developed a new approach where two types of monohead glycolipids, active sialic acid-β-glucoside (G1) and inactive lactose- β-glucoside (G2), are embedded onto the surface of a polydiacetylene (PDA) vesicle to mimic native glycolipids on the cell surface. Vesicles prepared in this manner show good selectivity with a 10 ng/mL detection limit and 5 min response time to Hemagglutinin (HA1), which is more sensitive than any HA1 biosensors ever known. Moreover, in the formation of color-changeable vesicles, a very strong synergistic effect between G1 and G2 has been found, offering a novel strategy to construct effective biosensor receptors, as well as a new way to study the surface combination effect that is potentially important to the immunology study of epidemic disease.

INTRODUCTION

Fast and sensitive detection of epidemic viruses is of the utmost importance for human beings nowadays. A commonly used approach is to mimic biological interfaces upon which specific molecular events could occur (*1*). Such interfacial biological processes have been found to be detectable with the help of a polydiacetylene (PDA) vesicle (*2*−*4*).

Many efforts have been devoted to developing PDA-based vesicles for detection of various biologically important analytes, such as lipophilic enzymes (*5*) (*6*), antibacterial peptides (*7*), mammalian peptides (*8*), ions (*9*), antibodies (*10*−*12*), proteins (*13*), and pharmacologically active compounds (*14*). It is generally believed that the PDA vesicle is a good model system to mimic the cell surface in the study of the surface conjugation events. To the best of our knowledge, however, there are only a few studies on the detection of the key polysaccharide or carbohydrate−protein interaction (*15*−*17*), although it is one of the most widely existing and important types of conjugation in cell membranes. Furthermore, almost all the epidemic virus invading processes involve polysaccharides on cell surfaces. One major limitation to the PDA-based approach is the difficulty of synthesizing complicated probes that contain more than one saccharide group in a molecule as effective binding sites. If these obstacles could be overcome by self-assembly technology, for example, by inserting receptors and various saccharide groups into the same vesicle to make it analogous to natural complex polysaccharide receptors on the cell, a much easier approach for biosensor design and construction would be developed, which may lead to a wide range of applications.

Herein, we report an approach based on the strategy mentioned above. The recognition element assembled in the colorimetric mixed PCDA sensor consists of sialic acid-β-glucoside (G1) and lactose-β-glucoside (G2) receptors, both having a tetraethylene glycol spacer and C12 hydrocarbon. The fragment of influenza virus H5N1 protein of Hemagglutinin (HA1) on the cell surface is used to act as the target for detection.

EXPERIMENTAL PROCEDURES

Materials. 10,12-pentacosadiynoic acid (PDA) was purchased from Lancaster Co. in 98% purity, which was further purified by dissolving in chloroform and then filtered to remove polymerized monomers before use. Dimyristoylphosphatidylcholine (DMPC) was purchased from Sigma, and bovine serum albumin (BSA) was purchased from Beijing Reagent Co. *E.coli DH5*α was provided by Institute of Zoology of the Chinese Academy of Sciences. Sialic acid-acid-β-glucoside (G1) and lactose-β-glucoside (G2) receptors were synthesized according to previously reported procedures (*18*−*24*). The fragments of the influenza virus protein of Hemagglutinin on the cell surface (HA1) were obtained as reported (*25*−*29*), with details of abstraction and synthesis procedures given in the Supporting Information (SI).

Preparation of PDA Mixed Vesicles. The PDA vesicles were prepared according to literature procedures (*12, 17*). The typical procedure for the mixed vesicles preparation is as follows. The mixed components (PDA, DMPC, G1, G2) with various molecular fractions were dissolved in $CHCl_3$ followed by solvent evaporation. After adding 0.01 mol/L phosphate buffer (pH 6.9), the suspension (1 mmol L^{-1} of the mixed component) was probe sonicated for 5−6 min at approximately 80 °C. After the sonication, the solution was cooled to room temperature and then stored overnight at 4 °C to induce crystallization of lipid membranes. Polymerization was carried out under irradiation at 254 nm wavelength for 10 s. All vesicles used in our experiment have the same DMPC to PDA ratio of 4:6. The ratios of G1 and G2 in the vesicles were 1:0, 9:1, 7:3,

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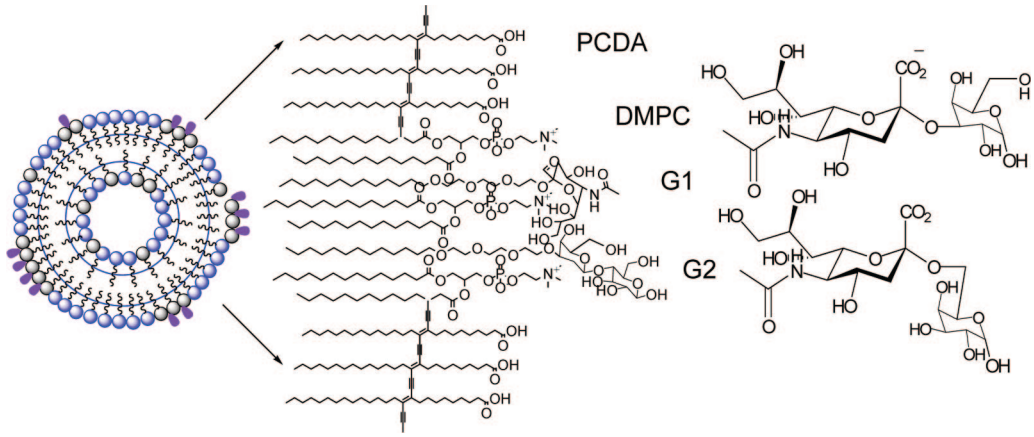

Figure 1. Schematic structure of the PDA vesicle functionalized with glicolipids: Receptors/DMPC/PDA assemblies (left) and the natural receptors found in the cell surface: α-Neu5Ac(2−3)-β-Gal,α-Neu5Ac(2−6)-β-Gal (right).

5:5, 3:7, 1:9, and 0:1, respectively, and the total molar ratio of (G1+G2) to PDA to DMPC is 0.2:6:4.

UV−vis Spectroscopy. Measurement of UV−vis spectra for the mixed lipid vesicles in water were carried out using a two-beam Hitachi U2800 UV−vis spectrometer. The pH of the vesicle solutions was maintained at 6.9 with phosphate buffer. During the measurement, a certain amount of aqueous analyte solution was injected stepwise into the vesicle solution with a syringe. All measurements were carried out at 35 °C.

Colorimetric transition from blue to red was monitored quantitatively by the colorimetric response (CR), which is defined as (*2, 11, 17*)

$$CR = [(PB_0 - PB_f)/PB_0] \times 100\% \quad (1)$$

where $PB = A_{blue}/(A_{blue} + A_{red})$, and A_{blue} and A_{red} are the absorbances of the blue component ($\lambda_{max} \approx 650$ nm) and the red component ($\lambda_{max} \approx 540$ nm) in the UV−vis spectrum; PB_0 and PB_f are the initial and final percentages of the blue component in the vesicle solution before and after adding the analyte, respectively.

RESULTS AND DISCUSSION

The design of a new system for rapid detection of interfacial biomolecular interactions in general, and receptor−ligand recognition in particular, has to fulfill two main objectives. First, the system structure should allow the interaction event to take place readily. Second, the specific interactions between the receptor and the ligand should be easily transformed into a quantified chemical or physical signal, such as transferring the conjugation event into a color change. The Vesicle-(G1+G2) we have designed, shown schematically in Figure 1, satisfies the above two requirements.

We synthesized glycolipids with sialic acid (G1) and lactose (G2), respectively, and designed the headgroups pointing outward from the PDA vesicle surface in order to assemble them to form an analogue to the complicated influenza virus H5N1 receptors where sialic acid linked to galactose by α-2,3 linkage [α-Neu5Ac(2−3)-β-Gal] and α-2,6 linkage [α-Neu5Ac(2−6)-β-Gal], the minimal recognition site for the HA1 (*25*). In our experiment, galactose is replaced by lactose, because the lactose is composed of two galactoses so that a "trap" with sialic acid to HA1 can be formed. In order to make the glycolipids extend in water, a tetraethylene glycol spacer was chosen as linker; G1 and G2 both ended with a C_{12} hydrocarbon chain for the immobilization to the vesicles.

It has been found that local dimyristoylphosphatidylcholine (DMPC) domains incorporated into PDA vesicles not only increase the detection sensitivity, but also open a new avenue

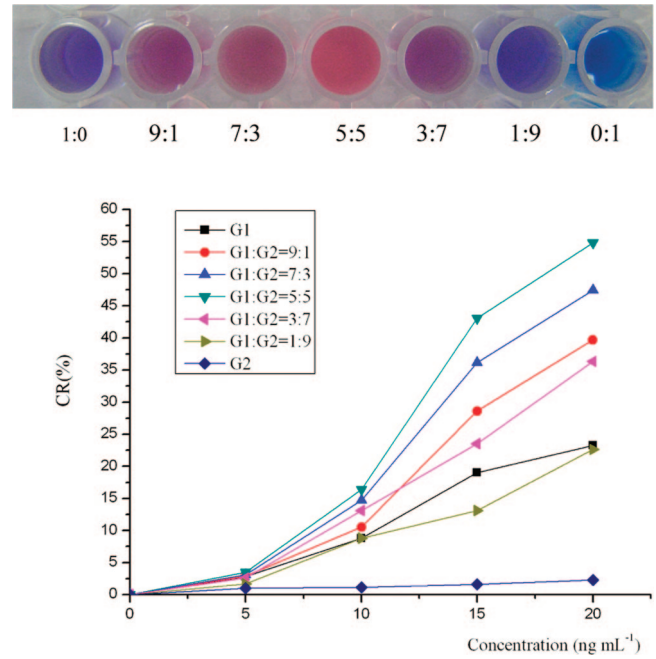

Figure 2. The color of the seven different vesicles with 20 ng mL^{-1} HA1, from left to right, with the ratio of G1 to G2 indicated (up), and the CR% of seven mixed vesicles with addition of different amount of HA1(down).

for lipophilic biological molecules physically inserting the mixed vesicles. DMPC was used as a component of vesicles. The molar ratio of glycolipid/DA/DMPC equals 0.2:6:4, where the glycolipids can be G1, G1+G2, or G2. A series of vesicles were prepared to study how the combined glycolipids affect the conjugation. In these vesicles, the ratio of G1 to G2 in the vesicles changes in the following way: 1:0, 9:1, 7:3, 5:5, 3:7, 1:9, and 0:1. After photopolymerization, the PDA mixed vesicles appeared to be clear blue to the naked eye and exhibited a maximal absorption at 640 nm, which is due to their backbone alternating ene−yne conjugated structure.

According to our experiment, the larger the concentration of DMPC in the vesicle, the more sensitive the PDA vesicle will be. However, if the amount of receptor G1+G2 or DMPC exceeds a certain threshold value, the blue-colored PDA vesicle could not be observed, which is believed to be due to the destruction of the effective conjugation length of the PDA backbone.

In Figure 2, the color of PDA vesicles functionalized with G1, G2, or G1+G2 shaded from blue into purple, amaranth,

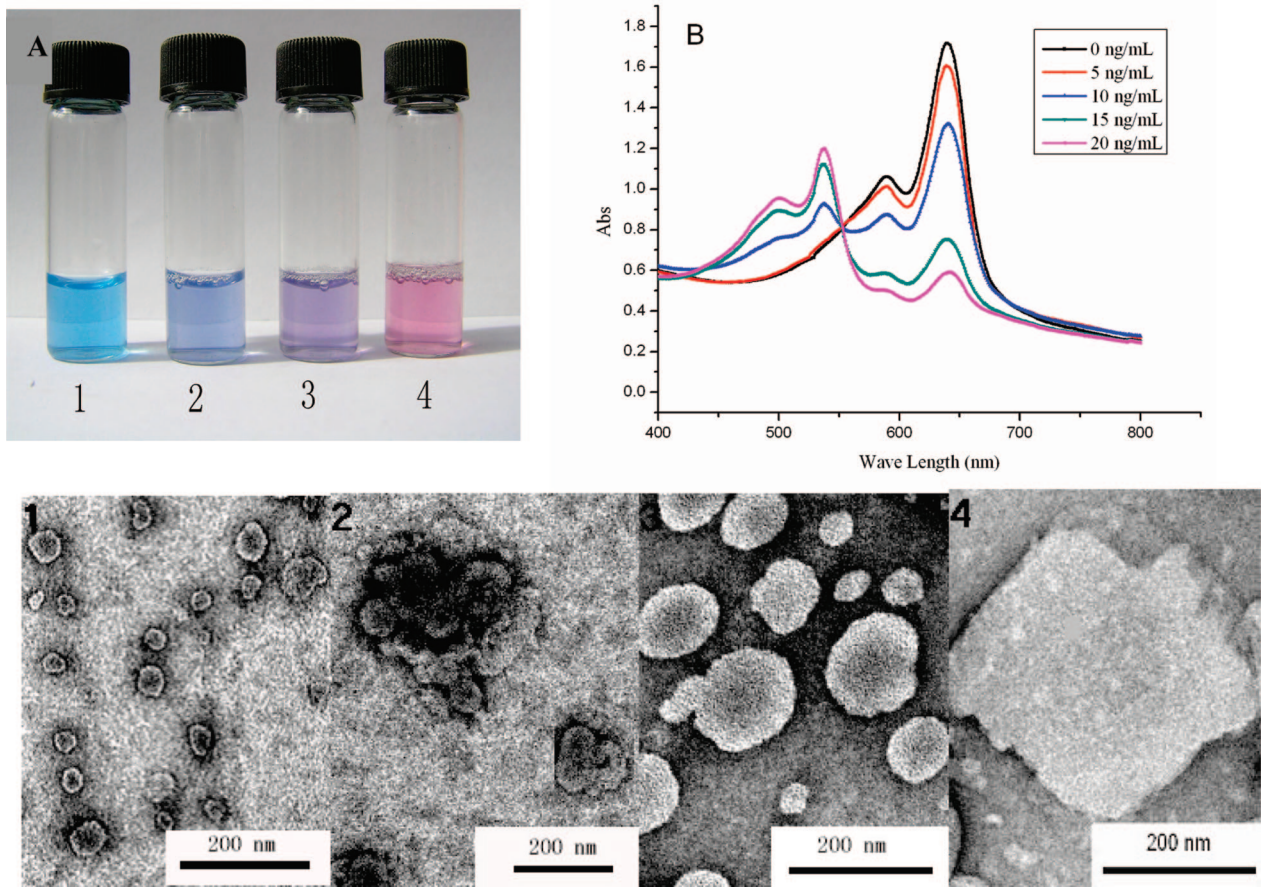

Figure 3. Interaction of vesicle-(G1+G2 = 5:5) with different amounts of HA1 in solution. (A) Photographs. (1) Control without HA1. (2) 5 ng mL^{-1}. (3) 10 ng mL^{-1}. (4) 20 ng mL^{-1}. (B) Visible adsorption spectra of vesicle-(G1+G2 = 5:5) solution mixed with HA1. (C)TEM images of vesicle-(G1+G2 = 5:5) solution tested with different concentration of HA1: (1) control. (2) 5 ng mL^{-1}. (3) 15 ng mL^{-1}. (4) 20 ng mL^{-1}.

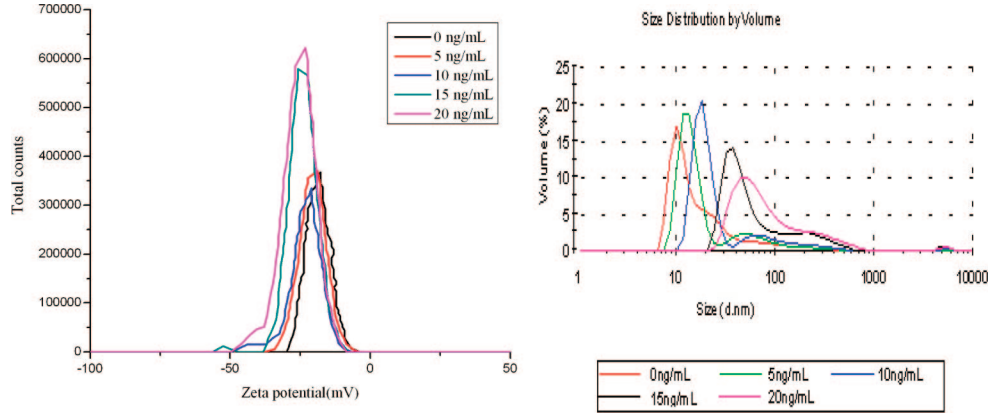

Figure 4. ζ potential and volume distribution of vesicles measured by using a Zetasizer, Nano ZS Malvern Instruments Ltd.

magenta, and red when the surface-assembled receptor was bound to different amounts of HA1. It is intriguing that the degree of the color change of the polymerized vesicles functionalized with G1 or G2 alone was much less than those functionalized with (G1+G2) when HA1 was added; vesicle-G1 underwent a color change from blue to purple as the HA1 concentration reached 20 ng mL^{-1}, whereas the color of vesicle-G2 still remained blue, meaning no conjugation occurred at all. On the other hand, vesicle-(G1+G2), especially G1/G2 = 5:5, causes a remarkabe color change even at a very low concentration of HA1 (ca. 10 ng mL^{-1}), showing a very strong synergistic effect for the recognition of virus HA1. This behavior can also be found in the change of CR% of seven mixed vesicle, as shown in Figure 3, where the value of CR is ordered as follows: vesicle-(G1/G2 = 5:5) > vesicle-(G1/G2 = 7:3) > vesicle-(G1/G2 = 3:7) > vesicle-(G1/G2 = 9:1) > vesicle-G ≈ vesicle-(G1/G2 = 1:9) > vesicle-G2 when 10 ng mL^{-1} HA1 was added. However, the value of CR of the vesicle-(G1/G2 = 9:1) is higher than that of the vesicle-(G1/G2 = 3:7) when HA1 was added from 15 ng mL^{-1} to 20 ng mL^{-1}. These results show that there is some synergistic effect between G1 and G2. It was reported previously (*2*) that lactose (receptor G2) is not a ligand for the hemagglutinin lectin, whereas sialoside (receptor G1) is the selective and sensitive one. To the best of our knowledge, this kind of synergistic effect and high sensitivity have not yet been reported previously.

Figure 3A,B shows the relationship between the color change and the visible absorption spectra of the vesicle-(G1/G2 = 5:5)

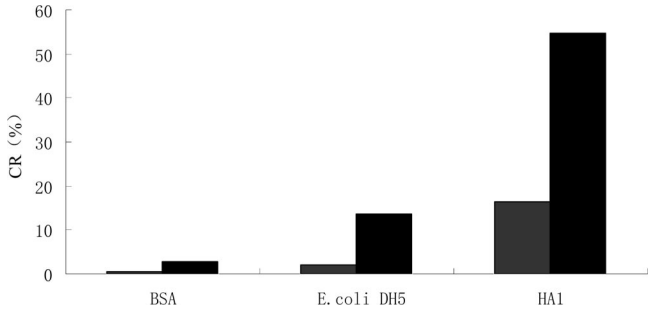

Figure 5. Absorbance difference at 640 nm of vesicle-(G1+G2) changed with different species of biomolecules: (1) BSA. Left: 10 ng mL^{-1}. Right: 25 ng mL^{-1}. (2) *E.coli DH5.* Left: 10 ng mL^{-1}. Right: 25 ng mL^{-1}. (3) HA1. Left: 10 ng mL^{-1}. Right: 20 ng mL^{-1} within 5 min.

as it encountered HA1. When the color changed, the absorption maximum at 640 nm decreased with a concurrent absorption increase in the maximum at 540 nm in visible absorption spectra. It is commonly accepted that the blue−red chromatic transitions observed in PDA can be attributed to the disturbance of the conjugation length of the polydiacetylene backbone. Our experiment showed that binding of the viral hemoglotinen to the glycolipids affects the vesicle conformation to a great extent, which was clearly demonstrated in the TEM picture (Figure 3C). TEM images demonstrated that the vesicle packing shape after recognition with HA1 is much different from that of its original form. As more HA1 adsorbed on the vesicle surface, the size of vesicles-(G1+ G2) grows bigger, accompanied by the shape change due to fusion from spherical to flat rectangular. This occurs presumably because of the interaction between the HA1 proteins on the vesicle surfaces.

Two interesting results were found in these experiments: (1) It is possible to form a highly sensitive biosensing element in the vesicle surface simply by inserting G1 and G2 into the PDA vesicle, thus providing an easy way to mimic the receptors containing multiple saccharide complex on the cell surface. (2) A very strong synergistic effect between sensitive receptor (G1) and nonsensitive receptor (G2) was observed, indicating the importance of the receptor combination effect for the ligand conjugation on the vesicle surface that was less known previously, thus providing us an open area for further investigation.

To gain a deeper insight into the property of the vesicle surface, electrophoresis experimentation for the ζ potential measurement was carried out. It was found that, after adding more HA1, the ζ potential of the vesicles-(G1+G2) gradually changed to a more negative and constant value as shown in Figure 4. In combination with the volume changing in TEM pictures showed in Figure 3, it is reasonable to assume that after conjugating with HA1, the receptor surface was gradually covered by HA1 and reached a constant ζ value mainly due to HA1 no matter how large the vesicle was. TEM and CR measurements show that the agglomeration of particles still continued by the increase of HA1 concentration caused by the interaction between proteins on the vesicle surfaces or protein fragments in the bulk, which further made the aggregate volume and morphology change, and caused CR% to increase, respectively.

In order to further assess the color response due to nonspecific adhesion of the vesicles, some large biomolecules such as *E.coli DH5*α and bovine serum albumin were tested as controls. It appears that these molecules do not cause any vesicle color change even for a long time (at least 5 min) as shown in Figure 5. This is completely different from the case of vesicle-(G1+G2), whose color change from deep blue to amaranth or red may occur within less than 5 min when treated with HA1 10 ng mL^{-1} or 20 ng mL^{-1}. Together, our results show that vesicles-(G1+G2) prepared in this manner are promising candidates for biosensors. It shows very good specificity, high sensitivity, and short respondent time, thus being of great significance in its application in influenza virus H5N1 diagnosis.

CONCLUSIONS

This study describes an approach to the preparation of an effective biosensor for HA1 recognition on a chromatic vesicle surface based on a molecular assembly technique. It overcomes the limitation of current synthesis methods, which were known to be both complicated and difficult. The colorimetric assay is robust and easy to carry out, yielding results that are much faster and more sensitive than commonly used traditional biochemical techniques. Although the exact mechanism of the synergistic effect is not clear yet, the phenomenon itself has already shown great significance in application. Further study of the mechanism of this finding and its applicability by assembling glycolipids to improve the sensitivity in bioconjugate events shall be an attractive area in the future. Our results also show that, in combination with the self-assembly technology, the chromatic vesicle protocol could be utilized as a platform for various biochemical applications, such as for new drug metabolism and for pharmacokinetics of the H5N1 virus, including the establishment of a receptor library for disease diagnostics.

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Supporting Information Available: Experimental procedures, synthesis of sialic acid-β-glucoside (G1) and lactose-β-glucoside (G2), expression and purification of HA1 and preparation of PDA mixed vesicles. This material is available free of charge via the Internet at http://pubs.acs.org.

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