SUPPORTING INFORMATION

Membrane Environment Can Enhance the Binding of Influenza A Virus Hemagglutinin to Cell Surface Glycan Receptors

Lei Shen,[1] Yini Wang,[2] Chia-I Lin,[3] Hung-wen Liu,[3] Athena Guo,[2] X.-Y. Zhu[1]

[1] *Department of Chemistry, Columbia University, New York City, NY 10027*
[2] *Microsurfaces, Inc., 1 West Forest Ave., Englewood, NJ 07631*
[3] *College of Pharmacy, University of Texas at Austin, Austin, TX 78712*

Materials & Synthesis

The HA protein, cloned and expressed from the most pathogenic H5N1 model A/Vietnam/1203/2004 (Viet04), was purchased from Prospec-Tany TechnoGene Ltd. (Israel). Egg phosphatidylcholine (EggPC), 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphoethanolamine-N-hexanoylamine (16:0 Caproylamine PE) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-succinyl (16:0 Succinyl PE) lipids were purchased from Avanti Polar lipids (Alabaster, AL, USA). Dibenzyl cyclootyne (DBCO) and N-hydroxysuccinimide (NHS) based bifunctional linking molecules with or without oligoethylene glycol spacers (DBCO-PEG_5-NHS ester & DBCO-NHS ester) were purchased from Click Chemistry Tools (Scottsdale, AR, USA). The bifunctional oligoethylene glycol spacer, H_2N-PEG_4-COOH, was purchased from Broadpharm (San Diego, CA, USA). All tri-saccharides with azide (-N_3) tags were provided by the Consortium for Functional Glycomics. Tris(hydroxymethyl)aminomethane (Tris) and phosphate buffers were freshly prepared from NaCl, Tris and sodium phosphate salts to give pH 7.4 at 25 ^oC. All other reagents and solvents were purchased from Sigma Aldrich (USA).

Synthesis of DBCO-OEG-lipid (ϕ-OEG-lipd). To a solution of 16:0 Caproylamine PE lipid (5.2 mg, 6.4 micro-mole) and DBCO-PEG_5-NHS (4.5 mg, 6.4 micro-mole) in $CHCl_3$ (0.64 ml) was added triethylamine (1.9 mg, 19.2 micro-mole). The solution was stirred overnight at room temperature. After removal of the solvent, the product was used without further purification. Characterization of the product (in methanol) by matrix-assisted laser desorption ionization (MALDI) mass spectrometry gave a molecular mass of 1383.3±0.5 atomic units (AU); the calculated mass from the molecular formula was 1383.8 AU.

Synthesis of DBCO-lipid (ϕ-lipd). To a solution of 16:0 Caproylamine PE lipid (5.2 mg, 6.4 micro-mole) and DBCO-PEG_5-NHS (2.6 mg, 6.4 micro-mole) in $CHCl_3$ (0.64 ml) was added triethylamine (1.9 mg, 19.2 micro-mole). The solution was stirred overnight at room temperature. After removal of the solvent, the product was used without further purification. Characterization of the product (in methanol) by MALDI mass spectrometry gave a molecular mass 1092.7±0.5 AU; the calculated mass from the molecular formula was 1092.4 AU.

Conjugation of glycan-azides to ϕ-OEG-lipd. To a solution of ϕ-OEG-lipd (0.56 micro-mole) in DI water was added each glycan-azide (0.28 micro-mole), with a total solution volume of 80 µl. The mixture was stirred overnight at room temperature and then lyophilized under vacuum. The final products were characterized by MALDI mass spectrometer. **33**-ϕ-OEG-lipid (found 2127.2±0.5 AU, calculated 2127.4 AU); **36**-ϕ-OEG-lipid (found 2127.2±0.5 AU, calculated 2127.4 AU); **32**-ϕ-OEG-lipid (found 2152.6±0.5 AU, calculated $M + 3Na^+ - 2H^+ = 2152.4$ AU); **35**-ϕ-OEG-lipid (found 2152.6±0.5 AU, calculated $M + 3Na^+ - 2H^+ = 2152.4$ AU); **Man**-ϕ-OEG-lipid (found 1632.3±0.5 AU, calculated 1633.0 AU). Fuc-ϕ-OEG-lipd (found 1616.5±0.5 AU, calculated 1617.0 AU). The structures of all glyco-lipids are shown in **Scheme 1**.

Synthesis of HOOC-OEG-lipid. To a solution of 16:0 Succinyl PE (44 mg, 54 micro-mole) in $CHCl_3$ (5.4 ml) was added 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (13 mg, 70 micro-mole) and NHS (6.2 mg, 54 micro-mole). The mixtrure was stirred overnight at room temperature and then concentrated in vacuum. The residue was re-dissolved in $CHCl_3$ (2.7 ml) at room temperature. H_2N-PEG_4-COOH (14 mg, 54 micro-mole) and triethylamine (5.5 mg, 54 micro-mole) were added and the solution was stirred overnight. The solvent was removed under vacuum to give the final product. MALDI mass spectrometry analysis gave a molecular mass of 1039.9±0.5 AU and the calculated mass was $M+H^+ = 1039.5$.

Reduction of glycan-azide. An aqueous solution (5 ml) of **33**-N_3 or **36**-N_3 (1.75 mg) and 5% Pd/C (5 mg) was stirred under hydrogen atmosphere (1 atm) for 3 h. The reaction mixture was filtered through a Celite pad (pre-washed with DI water, methanol, and then air-dried). The filtrate was freeze-dried to yield the product. Electro-spray ionization (ESI) mass spectrometry analysis of **33**-NH_2 or **36**-NH_2 gave a molecular mass of 740.3 AU; the calculated mass ($M + Na^+$) was 740.3 AU.

Conjugation of amino-glycan to HOOC-OEG-lipid: To a solution of HOOC-OEG-lipid (15 mg, 18 micro-mole) in $CHCl_3$ (1.8 ml) was added EDC (4.4 mg, 23 micro-mole) and NHS (2.1

mg, 18 micro-mole). The solution was stirred overnight at room temperature and the solvent was evaporated. The resulting NHS ester (0.26 mg, 0.28 micro-mole) was re-dissolved in $CHCl_3$ (28 µl) at room temperature. **33**-NH_2 or **36**-NH_2 (0.2 mg, 0.28 micro-mole) was dissolved in DMSO (28 µl) and added to the NHS ester solution. Triethylamine (0.028 mg, 0.28 micro-mole) was added and the mixture was stirred for 4 h at room temperature. The solvents were removed by lyophilization to yield the glycan-PEG-lipid product. MALDI mass spectrometry analysis: **33**-OEG-lipid, found 1761.9±0.5 AU, calculated ($M + H^+$) 1761.6; **36**-OEG-lipid: found 1806.7±0.5 AU, calculated ($M+2Na^+$) 1806.6 AU.

Supported Lipid Bilayer Microarrays

We developed a microarray surface for label-free analysis by surface plasmon resonance imaging (SPRi). As illustrated in Fig. S1A, we first deposit a 50 nm Au film (with a 2 nm Cr adhesion layer, not shown) on a glass coverslip in a metal thermal evaporator. This is followed by the deposition of another 2 nm Cr adhesion layer (not shown), followed by the deposition of a 10 nm SiO_2 film by plasma-enhanced chemical vapor deposition (PECVD). We then used photolithography, Cr thin film deposition (25 nm), and lift-off to define an array of SiO_2 holes (1 mm diameter each), with 0.6 mm spacing in a square lattice. The SiO_2 terminated holes, surrounded by a Cr barrier,[1] are used for the formation of supported lipid bilayer (SLB) arrays. Fig. S1B shows an SPR image of the sensor array. The dark area is the SiO_2 array and bright area is the Cr barrier. Analysis by X-ray photoelectron spectroscopy confirmed the presence of the SiO_2 surface surrounding by Cr matrix.

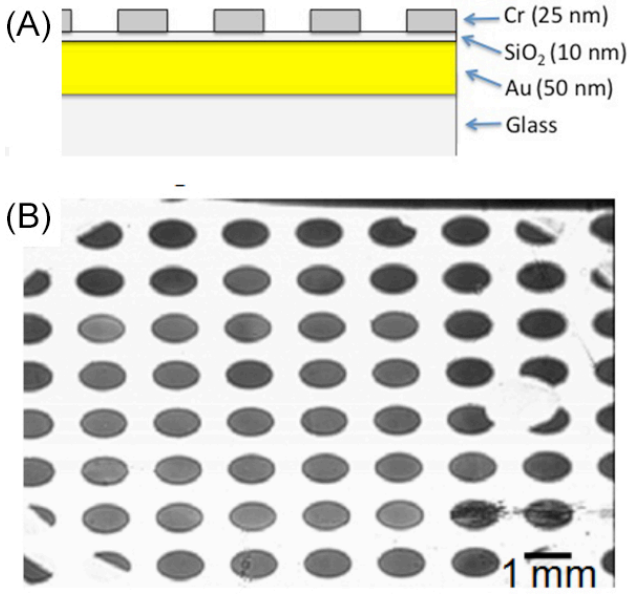

Figure S1. Top: cross-sectional illustration of the SPRi array sensor. Bottom: SPRi image of microarray chip. The dark circles are the SiO_2 surfaces and the bright area is the Cr barrier. The diameter of each spot is 1.0 mm.

Supported lipid bilayer (SLB)

The SLB microarrays containing functional glycans were generated within each SiO_2 spot on the SPRi sensor surface. Briefly, lipid mixtures containing eggPC and a small percentage of specific functional glycan-lipids were prepared in $CHCl_3$, dried, and re-suspended in 50 mM Tris buffer (pH=7.4) containing 25 mM NaCl to a total lipid concentration of 1.0 mg/ml. Suspension of the lipid mixture after pre-filtration was forced through a polycarbonate filter with 50 nm pores 11 times to yield a small unilamellar vesicle (SUV) solution with nominal diameters of ~100 nm. The SUV solutions were drop deposited onto the SiO_2 arrays and incubated for one hour at room temperature under controlled humidity. We removed excess vesicle solutions from the sensor surface by rinsing in Tris buffer.

Fluorescence recovery after photo-bleaching (FRAP)

To verify the fluidity of the supported lipid bilayer (SLB), we carried out measurement using fluorescence recovery after photo-bleaching (FRAP) on a fluorescence microscope. We mixed 0.05% of dye-tagged lipid, Texas Red tagged dihexadecanoyl-phosphatidylethanolamine (TR-DHPE) (Invitrogen, Carlsbad, CA), into the lipid mixture for SLB formation. An oval-shaped spot (~110 µm x 80 µm) was bleached by a laser beam (532 nm) for a few seconds. Fluorescence images were recorded (excitation at 435 nm and detected at 546 nm) at different times after photobleach. The fluorescence intensity within the bleached area is integrated and plotted as a function of time, as shown in Fig. S2. Fitting the data points with a two-dimensional diffusion model gives a lipid diffusion constant of 0.8±0.2 $\mu m^2/s$, as expected for SLBs on the hydrophilic SiO_2 surface.[2,3]

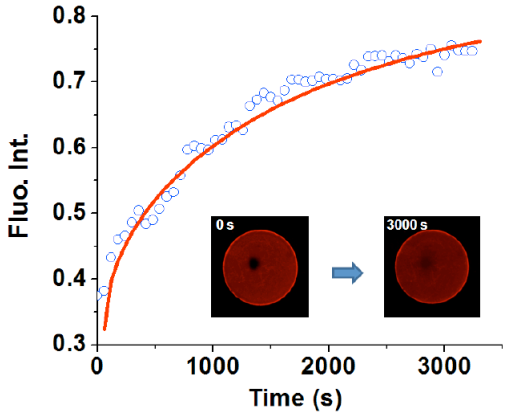

Figure S2. Fluorescence intensty (circles) as a function of time following photobleaching of an SLB spot containing 99.15% EggPC, 0.8% **33**-ϕ-OEG-lipid, and 0.05% TR-DHPE. The inset shows images taken at 0s and 3000s, respectively, after photobleaching. Fitting (red curve) of the FRAP data to a standard two-dimensional diffusion model gives a diffusion constant of 0.8 ± 0.2 $\mu m^2/s$. The total recovery of fluorescence intensity at long times is about 90%.

Surface Plasma Resonance (SPR) imaging

We carried out label-free protein-glycan binding measurements using a commercial SPR instrument (SPRImager II, GWC Technologies Inc., Madison, WI) at room temperature (25 ^{o}C). The Au SPR sensor surface was in contact with a liquid flow cell. The sample was initially equilibrated with the sodium phosphate buffer (pH 7.4) solution at a flow rate of 3 μL/s. Once a stable background was obtained, HA protein phosphate buffer solution (400 nM) was injected into the cell at the same flow rate. The SPR signals from different spots (1.0 mm diameter each) on the array were averaged (x4) and recorded as a function of time. After a fixed time of protein adsorption, a phosphate buffer solution was injected into the cell to replace the protein solution and to wash away weakly adsorbed protein molecules.

In SPR measurement, the surface plasmon was excited by collimated polychromatic *p*-polarized light directed onto the gold sensor surface through a prism assembly at an angle of incidence θ. The intensity change (ΔI) measured at a constant angle is directly proportional to the change in the bulk refractive index of the solution near the sensor surface. The effective thickness (d) for the adsorbed layer is given by[4]

$$d = (l_d/2)[\Delta I/s(n_a-n_s)] \quad \text{(s1)},$$

where l_d is the decay length of the evanescent field near the gold surface (taken to be 37% of the wavelength of the light at 800 nm); s = 10.6/0.0006 is a sensitivity parameter for the instrument, which is calibrated by a H_2O/ethanol(1% volume) mixture; n_a is the refractive index of the HA protein, taken to be equal to the known value of 1.57 for bovine serum albumin (BSA) which has a similar molecular weight as HA; n_s = 1.35 is the refractive index of the solution. Based on the effective thickness determined in the experiment, we can estimate the surface coverage (C) of adsorbed molecules as:

$$C \text{ (molecules/nm}^2) = d/V \quad \text{(s2)}$$

where V is the specific volume which is 0.734 cm^3/g for HA in the solution.[5] This gives C = $3.271 \times 10^{-4} \times \Delta I$ (molecules/nm^2).

The Precursor Model

For precursor mediated interaction between an HA trimer (P) and membrane surface glycan receptors, the process can be described by the four step process in equation (1):

$$P \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} P^*$$

$$P^* + \underline{G} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \underline{PG}$$

$$\underline{PG} + \underline{G} \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} \underline{PG}_2$$

$$\underline{PG}_2 + \underline{G} \underset{k_{-4}}{\overset{k_4}{\rightleftarrows}} \underline{PG}_3 \qquad \text{(s3).}$$

The solution protein concentration under flow conditions can be approximated as an constant, $[P]_0$. The steady-state approximation for the precursor intermediate, P^*, is given by:

$$\frac{d[P^*]}{dt} = k_1[P]_0 - k_{-1}[P^*] - k_2[P^*][\underline{G}] + k_{-2}[\underline{PG}] = 0 \qquad \text{(s4)}$$

which gives

$$[P^*] = \frac{k_1[P]_0 + k_{-2}[\underline{PG}]}{k_{-1} + k_2[\underline{G}]} \qquad \text{(s5)}$$

where $[P^*]$, $[\underline{G}]$, and $[\underline{PG}]$ are membrane surface concentrations of the the precursor state, glycan receptor, and monovalent $\underline{PG}$ complex, respectively. The total rate of protein adsorption (on the membrane surface), which is measured in SPR, is given by

$$\frac{d[P_t]}{dt} = \frac{d\left([P^*] + [\underline{PG}] + [\underline{PG}_2] + [\underline{PG}_3]\right)}{dt} = k_2[P^*][\underline{G}] - k_{-2}[\underline{PG}] \qquad \text{(s6).}$$

We focus on the initial assocaition kinetics where the bound protein density is $\leq 10^{-3}$ /nm^2, which is more than one order of magnitude less than the density of glycan receptors $[\underline{G}] \sim 2\text{x}10^{-2}$/nm^2. Thus, $[\underline{G}]$ can be approximated by the starting glycan density, $[\underline{G}]_0$. We can make the further approximation that the initial concentration of monovalent $\underline{PG}$ is much higher than that of the mulitvalent $\underline{PG}_2$ or $\underline{PG}_3$. Under these short time approximations, as well as the steady-state approximation in equation (s5), equation (s6) becomes:

$$\frac{d[P_t]}{dt} \approx \frac{d[\underline{PG}]}{dt} \approx \frac{k_1 k_2 [P]_0 [\underline{G}]_0 - k_{-1} k_{-2} [\underline{PG}]}{k_{-1} + k_2 [\underline{G}]_0} \qquad \text{(s7).}$$

Integration of equation (s7) over time (t) gives:

$$[P_t] \approx [\underline{PG}] \approx \alpha \cdot \left[1 - \exp(-\beta \cdot t)\right] \qquad \text{(s8),}$$

with the two parameters given by

$$\alpha = \frac{k_1}{k_{-1}} \cdot \frac{k_2}{k_{-2}} \cdot [P]_0 \cdot [\underline{G}]_0 = K_1 \cdot K_2 \cdot [P]_0 \cdot [\underline{G}]_0 \quad \text{(s9)},$$

$$\beta = \frac{k_{-2}}{1 + [\underline{G}]_0 k_2 / k_{-1}} \quad \text{(s10)}.$$

Here t = 0 corresponds to time of injection of the protein solution. Equations (s8-10) are presented as equations (3-5) in the text.

For dissociation, the solution is switched to that of washing buffer under flow conditions and the solution protein concentration is approximated as $[P] \sim 0$ and the steady state approximation becomes:

$$[P^*] = \frac{k_{-2}[\underline{PG}]}{k_{-1} + k_2[\underline{G}]} \quad \text{(s11)}.$$

Substitute equation (s11) into equation (s6) gives the dissociation rate:

$$\frac{d[P_t]}{dt} \approx \frac{d[\underline{PG}]}{dt} \approx \frac{-k_{-1}k_{-2}[\underline{PG}]}{k_{-1} + k_2[\underline{G}]} \quad \text{(s12)}.$$

We apply the short time approximation when the dissociation rate from the monovalent $\underline{PG}$ is much higher than that from the mulitvalent $\underline{PG_2}$ or $\underline{PG_3}$. The concentrations of the latter can be taken as constants at short times. This approximation is supported by the fact that the equilibrium constants of multivalent interactions are many orders of magnitude higher than the corresponding monovalent interaction.[6] We make the further approximation that the concentration of surface glycan receptor, $[\underline{G'}]$, can be approximated as a constant at the short time limit. Under these short time approximations, equation (s12) can be integrated over time (t') to:

$$[\underline{PG}] \approx [\underline{PG}]_0 \cdot \exp(-\beta' \cdot t') \quad \text{(s13)},$$

and the total protein bound to the surface is given by:

$$[\underline{P_t}] \approx [\underline{PG}]_0 \cdot \exp(-\beta' \cdot t') + [\underline{PG_2}]_0 + [\underline{PG_3}]_0 \quad \text{(s14)},$$

where $[\underline{PG}]_0$, $[\underline{PG_2}]_0$, and $[\underline{PG_3}]_0$ are the surface concentrations of monovalent, bivalent, and trivalent HA-glycan complexes at the time of washing buffer injection (t' = 0) and β' is:

$$\beta' = \frac{k_{-1}k_{-2}}{k_{-1} + k_2[\underline{G}']} \quad \text{(s15)}.$$

Here, equations (s14) and (s15) are presented as equations (6) and (7) in the text.

The Direct Binding Model

In a conventional model for the interaction between an HA trimer (P) and membrane surface glycan receptors ($\underline{G}$), equation s3 is modified to:

$$P + \underline{G} \underset{k_d}{\overset{k_a}{\rightleftharpoons}} \underline{PG}$$

$$\underline{PG} + \underline{G} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \underline{PG_2} \qquad \text{(s16)}$$

$$\underline{PG_2} + \underline{G} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \underline{PG_3}$$

The solution protein concentration under flow conditions can be approximated as an constant, $[P]_0$. We focus on the initial assocaition kinetics where the bound protein density is $\leq 10^{-3}$ /nm^2, which is more than one order of magnitude less than the density of glycan receptors $[\underline{G}] \sim 2\text{x}10^{-2}$/nm^2. Under the short time approximation, the initial concentration of monovalent $\underline{PG}$ is much higher than that of the mulitvalent $\underline{PG_2}$ or $\underline{PG_3}$ and the reverse dissociation rate is much lower than the association rate, we have:

$$-\frac{d[\underline{G}]}{dt} = k_a[\underline{G}][P]_0 - k_d[\underline{PG}] \approx k_a[\underline{G}][P]_0 \qquad \text{(s17).}$$

Integration of equation (s17) over time (t) gives:

$$[P_t] \approx [\underline{PG}] \approx [\underline{G}]_0 \cdot \left[1 - \exp(-k_a \cdot [P]_0 \cdot t)\right] \qquad \text{(s18),}$$

which is equivalent to equation (s8).

For dissociation, the solution is switched to that of washing buffer under flow conditions and the solution protein concentration is approximated as $[P] = 0$. We apply the short time approximation when the dissociation rate from the monovalent $\underline{PG}$ is much higher than that from the mulitvalent $\underline{PG_2}$ or $\underline{PG_3}$. The concentrations of the latter can be taken as constants at short times. We have

$$-\frac{d[\underline{PG}]}{dt} = k_d[\underline{PG}] \qquad \text{(s19).}$$

Under such a short time approximations, equation (s19) can be integrated over time (t') to:

$$[\underline{PG}] \approx [\underline{PG}]_0 \cdot \exp(-k_d \cdot t') \qquad \text{(s20),}$$

which is equivalent to equation (s13).

Supporting Data

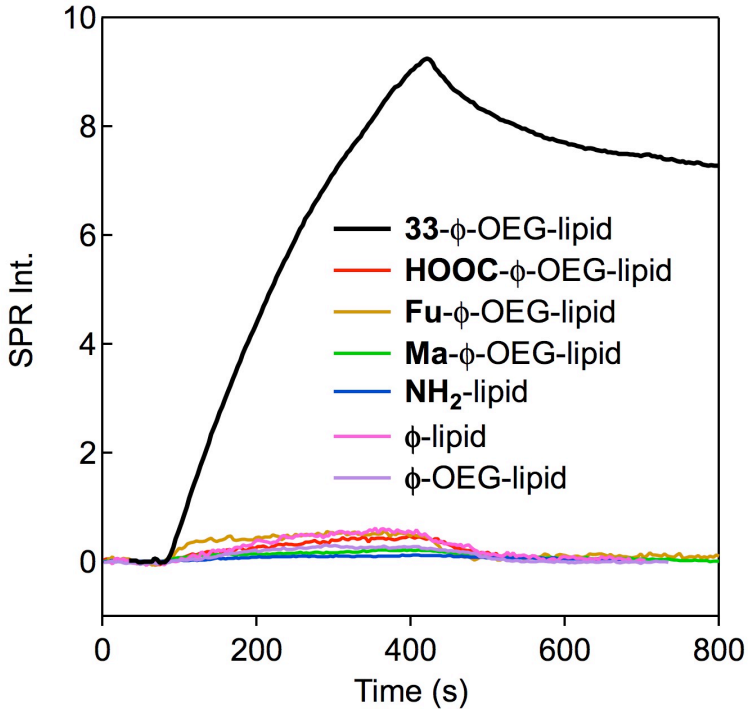

Figure S3. A comparison o SPR response for the binding of recombinant HA protein (400 nM) to α2-3 linked sialic acid, **33**-ϕ-OEG-lipid (black), with those to **HOOC**-ϕ-OEG-lipid, **Fu**-ϕ-OEG – lipid, **Ma**-ϕ-OEG –lipid, **NH_2**-lipid, **ϕ**-lipid, and **ϕ**-OEG-lipid on a supported lipid bilayer microarray. The concentration (mole percentage) of **33**-ϕ-OEG-lipid was 0.8% and those of all others were 4.0% in the supported lipid bilayers.

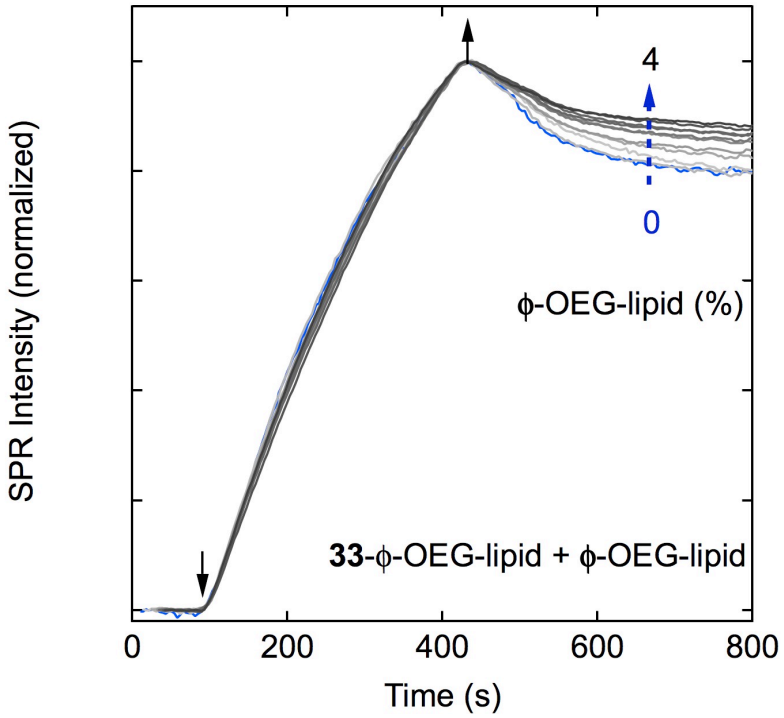

Figure S4. SPR responses for the interaction of HA protein (400 nM) with glycol lipids on an SLB array. The concentration of α2-3 linked sialic acid **33**-ϕ-OEG-lipid was kept constant (0.8 mol%), while the concentration of the second lipid molecule (ϕ-OEG-lipid) was varied from 0% (blue) to 0.05, 0.1, 0.3, 0.5, 0.8, 1.0, 1.5, 2.0, 3.0, and 4.0 mol% (grey with increasing darkness). This is a re-plot of Fig. 2A, with each curve normalized to the peak intensity. The black arrows mark the times of injection of protein solution and washing buffer, respectively. The blue dashed arrow shows ϕ-OEG-lipid with increasing concentrations.

References cited

s1 Mossman, K. & Groves, J. T. Micropatterned supported membranes as tools for quantitative studies of the immunological synapse. *Chem. Soc. Rev.* **36**, 46-54 (2007).

s2 Brian, A. A. & McConnell, H. M. Allogeneic stimulation of cytotoxic T cells by supported planar membranes. *Proc. Natl. Acad. Sci. U.S.A*, **81**, 6159-6163 (1984).

s3 Castellana, E. T. & Cremer, P. S. Solid supported lipid bilayers: From biophysical studies to sensor design. *Surf. Sci. Rep.* **61**, 429-444 (2006).

s4 Shumaker-Parry, J. S. & Campbell, C. T. Quantitative Methods for Spatially-Resolved Adsorption / Desorption Measurements in Real Time by SPR Microscopy. *Anal. Chem.* **76**, 907-917 (2004).

s5 Neuman, B. W.; Adair, B. D.; Burns, J. W.; Milligan, R. A.; Buchmeier, M. J. & Yeager, M. Complementarity in the Supramolecular Design of Arenaviruses and Retroviruses Revealed by Electron Cryomicroscopy and Image Analysis *J. Virol.* **2005**, *79*, 3822-3830.

s6 Mammen, M., Choi, S.-K. & Whitesides, G. M. Polyvalent interactions in biological systems: implications for design and use of multivalent ligands and inhibitors. *Angew. Chem. Int. Ed.* **37**, 2754-2794 (1998).