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Quantitative analysis of EGFR affinity to immobilized glycolipids by surface plasmon resonance

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ABSTRACT

EGF-induced activation of EGFR tyrosine kinase is known to be inhibited by ganglioside GM3, its dimer, and other mimetics. However, details of the interaction, such as kinetic properties, have not yet been clarified. The direct interaction is now defined by the surface plasmon resonance (SPR) technique. To determine the affinity of EGFR for lyso-GM3 or lyso-GM3 mimetic, these glycolipid ligands were covalently immobilized onto a sensor chip, and binding affinities were investigated. Results of these studies confirmed the direct interaction of lyso-GM3 or its mimetic with EGFR. A strong interaction between EGFR and lyso-GM3 or its mimetic was indicated by increased binding of EGFR to glycolipid-immobilized surface, in an EGFR dose-dependent manner.

1. Introduction

Glycosphingolipids, particularly gangliosides, are known to modulate growth factor receptor tyrosine kinase. A well-documented example is the inhibitory effect of GM3 on kinase associated with epidermal growth factor receptor (EGFR) in human epidermoid carcinoma A431 cells.[1–5]

The effects of GM3, lyso-GM3 dimer, and their mimetics, supplied as a sialylated lactoside, on various cancer cells expressing epidermal growth factor receptor (EGFR) were previously reported.[6,7] EGFR and GM3/lyso-GM3 dimer were shown to be co-expressed in the same membrane microdomain by (i) laser-scanning confocal microscopy, and (ii) direct interaction of EGFR with GM3-coated polystyrene beads. However, the kinetic properties and other details of such interactions remain unclear.

We investigated the kinetics of EGFR binding to lyso-GM3 or its mimetic. The interactions were analyzed with surface plasmon resonance (SPR). Recently, SPR was demonstrated as a useful technique to analyze binding specificity and affinity for interacting partners (antigen/antibody, glycoconjugate/lectin, etc.).[8–10]

The present study is focused on the following points: (i) quantitative interaction of EGFR with lyso-GM3/lyso-GM3 mimetic as an immobilized surface, (ii) quantitative interaction of MAM lectin, which recognizes a NeuAcα2-3Gal structure, with lyso-GM3/lyso-GM3 mimetic as an immobilized surface, and (iii) binding affinity of deglycosylated EGFR to lyso-GM3 as an immobilized surface.

2. Results

2.1. Chemical synthesis of the lyso-GM3 mimetic

Synthesis of the lyso-GM3 mimetic (compound **2**) is shown in Scheme 1. An –OH protected lyso-GM3 mimetic (**1**) was synthesized as described previously, and subsequent deacetylation yielded the lyso-GM3 mimetic (**2**), which was used as the immobilizing ligand in subsequent SPR studies.

2.2. Binding affinity of MAM for the lyso-GM3 or lyso-GM3 mimetic immobilized surface

To determine the properties of saccharides at the surface, we also studied the binding of *Maackia amurensis* lectin (MAM), which recognizes carbohydrate chains containing *N*-acetylneuraminic acid (sialic acid) residues α-(2→3) linked to galactose.[11,12]

Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; EGFR, epidermal growth factor receptor; ESIMS, electrospray ionization mass spectrometry; FBS, fetal bovine serum; GM3, NeuAcα3Galβ4Glcβ1Cer; MAM, *Maackia amurensis* lectin; PBS, phosphate-buffered saline; PNGase, peptide-N-glycosidase; SPR, surface plasmon resonance; RU, resonance unit; K_d, dissociation rate constants; K_a, association rate constants.

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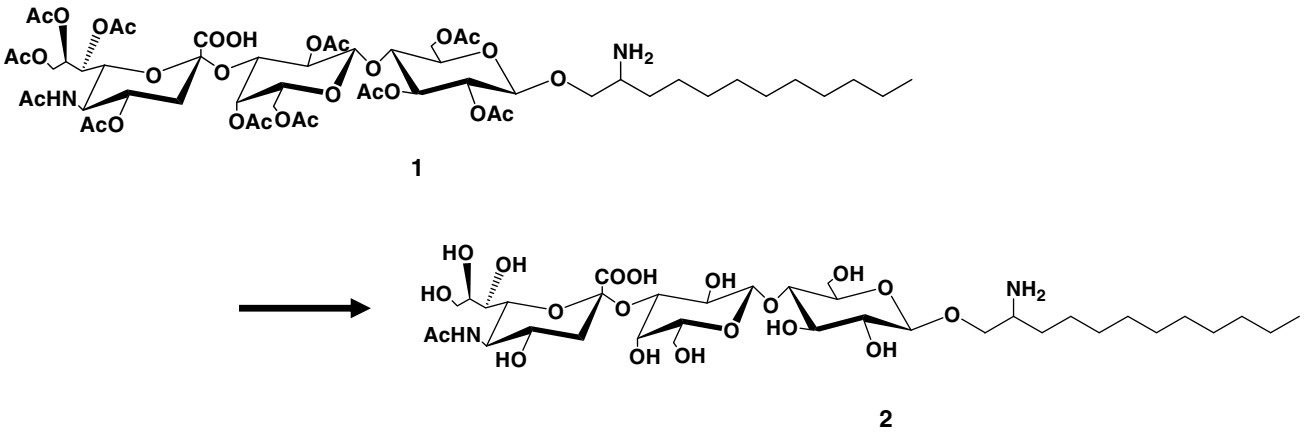

Scheme 1. Synthesis of a lyso-GM3 mimetic. Reagents and conditions: deacetylated in NaOMe, MeOH, rt, 15 h, 94.4% yield.

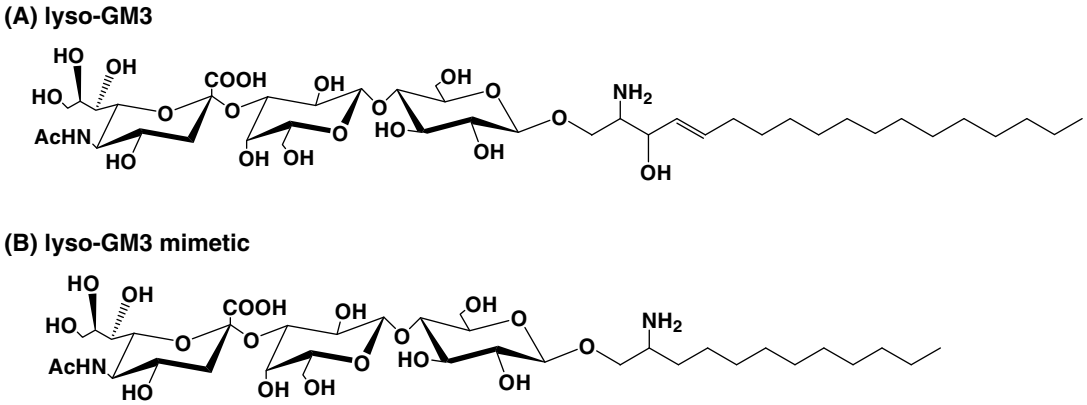

Figure 1. Structures of (A) lyso-GM3 and (B) lyso-GM3 mimetic.

A dose-dependent response was observed on injection with MAM (Fig. 2), and analysis of the equilibrium data for the interaction between MAM and lyso-GM3 or lyso-GM3 mimetic yielded a K_d of 3.96×10^{-7} M and 1.4×10^{-7} M, respectively.

2.3. Determination of binding kinetics for the interaction between EGFR and lyso-GM3 or its mimetic

EGFR showed specific binding to lyso-GM3 mimetic that was immobilized on a sensor chip (Figs. 3A and 4A). The signals increased in a dose-dependent manner. We evaluated the kinetic parameters $4.41 \times 10^5\ M^{-1}\ s^{-1}$ as k_{assoc} and $0.745\ s^{-1}$ as k_{dissoc}, for EGFR to lyso-GM3, and $8.09 \times 10^5\ M^{-1}\ s^{-1}$ as k_{assoc} and $0.835\ s^{-1}$ as k_{dissoc}, for EGFR to lyso-GM3 mimetic. From these values, the affinity constants K_a (apparent association constant) and K_d (apparent dissociation constant) were calculated as $5.92 \times 10^5\ M^{-1}$ (lyso-GM3) and $9.69 \times 10^5\ M^{-1}$ (lyso-GM3 mimetic), and 1.69×10^{-6} M (lyso-GM3) and 1.03×10^{-6} M (lyso-GM3 mimetic).

The degree of nonspecific binding of BSA on lyso-GM3 or lyso-GM3 mimetic surface was determined and overlaid to the sensorgram obtained at the same concentration of EGFR (Figs. 3B and 4B). The result clearly demonstrated the specificity of the interaction between EGFR and lyso-GM3 or its mimetic.

2.4. Deglycosylation of EGFR and its binding affinity for lyso-GM3 immobilized surface

The N-linked carbohydrate chains were removed from EGFR by recombinant PNGase F. EGFR was completely N-deglycosylated by incubation with PNGase F under native conditions, as shown in Figure 5A. Then, N-deglycosylated EGFR was subjected to SPR measurement without purification.

As shown in Figure 5B, PNGase F-treated EGFR showed a higher signal than control EGFR, while a nonspecific response was observed on injection with PNGase F, only. Increase of the response on the deglycosylated sample can be explained by considering the nonspecific binding of PNGase F mixed in the sample on the lyso-GM3 surface.

3. Discussion

Carbohydrate–protein interactions play key roles in many biological functions.[13–16] However, it is difficult to obtain detailed information on thermodynamic and kinetic parameters from analysis of a protein–carbohydrate complex, because the recognition of a simple saccharide by a protein (lectin, etc.) is usually in the low affinity range, and binding of saccharides to protein involves a variety of recognition processes.[9,10,17] On the other hand, complex oligosaccharides, as well as saccharide clusters, can strongly associate with proteins.[18–20] Various glycoclusters, for example, glycodendrimers, glyco-starburst, STARFISH, glycosylated peptides, and glycopolymer, have been reported.[20–24] In particular, a number of neutralizing agents for bacterial AB_5 toxins were developed, and their characteristics, including kinetic parameters, have been well studied.[25–28]

For GM3-containing saccharide clusters, a GM3-trimer[29] and a GM3-polymer[30–32] have been reported. A GM3-polymer was demonstrated to inhibit proliferation of NIH3T3 cells.[33] We previously

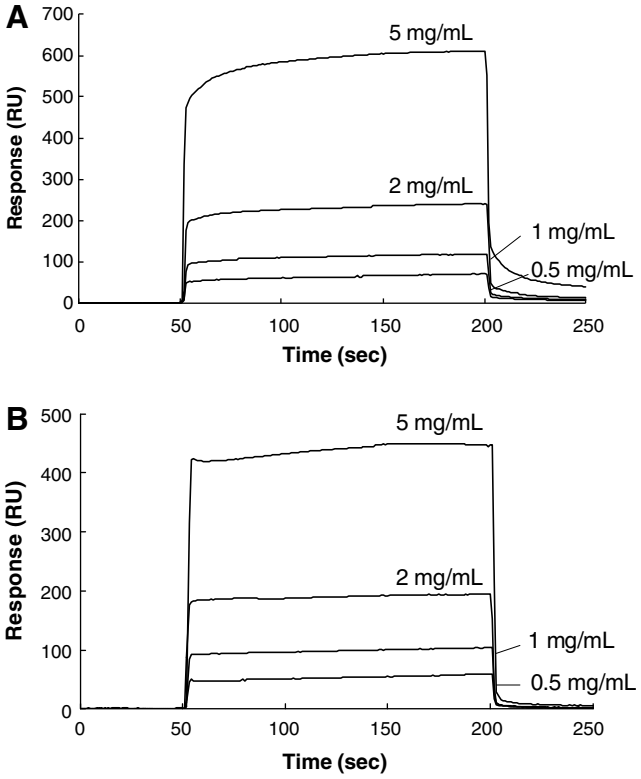

Figure 2. SPR sensorgram of different concentrations of MAM binding to (A) lyso-GM3 immobilized or (B) lyso-GM3 mimetic immobilized sensor chip. Aliquots of 50 μL of lectins at the indicated concentrations were injected, and running buffer was flowed over during the 100-s dissociation phase at a flow rate 20 μL/min.

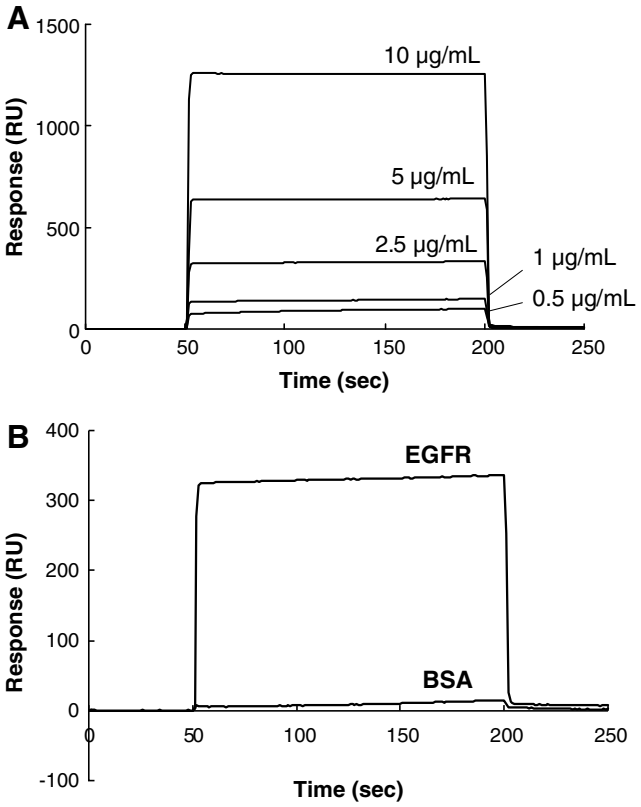

Figure 3. (A) Surface plasmon sensorgrams of EGFR to lyso-GM3 at various concentrations ranging from 0.5 to 10 μg/mL. (B) Overlay plots of EGFR and BSA observing the interaction to lyso-GM3 immobilized sensor chip surface at the same concentration (2.5 μg/mL). Flow rate = 20 μL/min, temp = 25 °C.

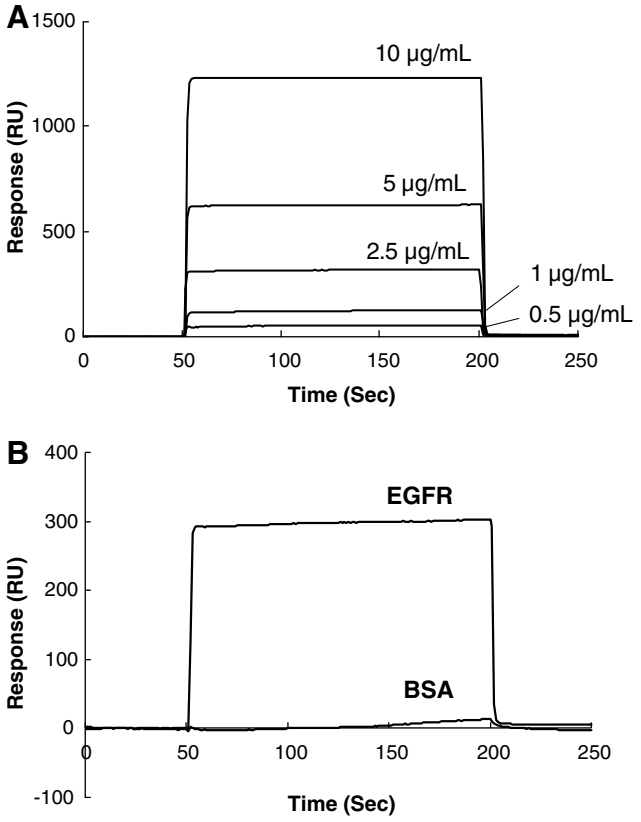

Figure 4. (A) Surface plasmon sensorgrams of EGFR to lyso-GM3 mimetic at various concentrations ranging from 0.5 to 10 μg/mL. (B) Overlay plots of EGFR and BSA observing the interaction to lyso-GM3 mimetic immobilized sensor chip surface at the same concentration (2.5 μg/mL). Flow rate = 20 μL/min, temp = 25 °C.

reported the biological effects of GM3 and its mimetics,[6,7] but details of physical properties were unclear. It is essential to demonstrate the specific binding of GM3 and its mimetics to EGFR.

The surface plasmon resonance (SPR) technique allows assessment of the specificity, strength, kinetics, and some thermodynamic parameters of two partners.[10] To determine the direct interaction between EGFR and GM3 or a GM3 mimetic, their lyso-types were synthesized as ligands. These lyso-glycolipid ligands were covalently immobilized onto a CM5 sensor chip, and binding affinities were investigated. In most previous studies, the glycolipid ligands were immobilized by hydrophobic interaction or self-assembled monolayers (SAMs).[9,17,19,34,35] In these methods there are many problems, such as lipid parts that are completely hidden in the surface of the hydrophobic sensor chip, shedding of ligands into flow, and difference of the lipid part from natural glycolipids. In the present study, lyso-GM3 or its mimetic was immobilized on a sensor chip with a covalent bond by an amine coupling method, since there was no need to synthesize S-introduced glycolipid for SAM, and the sensor chip ligand immobilized covalently can be re-used. Moreover, these ligands have amide bonds like the natural glycolipid by coupling onto the sensor chip and present lipid tails to the liquid phase.

The binding affinity of lectin MAM to lyso-GM3 or its mimetic is about the same order ($\sim 10^7$ M as K_d) as that of typical lectins to their partners.[36–39] The observed kinetic parameters (k_{assoc} and k_{dissoc}) and equilibrium constants (K_a and K_d) are shown in Table 1. These values reflect rapid association/dissociations and relatively weak binding affinities, which are typical of carbohydrates.[40] Results from SPR measurements indicate that the interaction between EGFR and lyso-GM3 or its mimetic is specific (Figs. 3 and

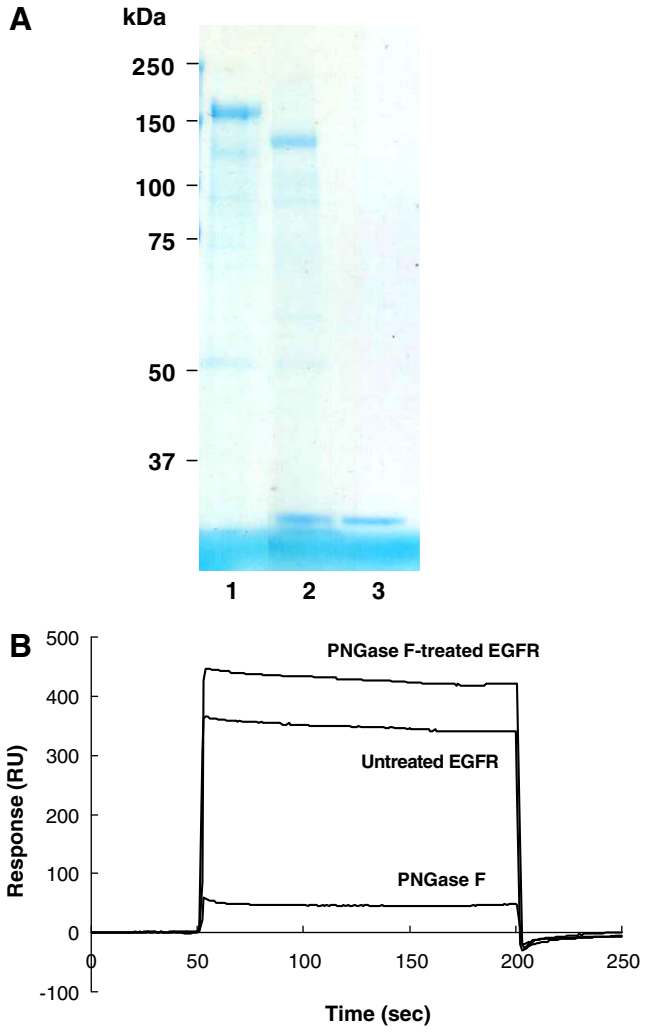

Figure 5. (A) SDS–PAGE gel of N-deglycosylated EGFR. Lane 1: untreated EGFR (N-glycosylated EGFR). Lane 2: PNGase F -treated EGFR (N-deglycosylated EGFR). Lane 3: PNGase F. (B) SPR sensorgram of glycosylated/deglycosylated EGFR or PNGase F, binding to lyso-GM3 immobilized sensor chip. Aliquots of 50 μL of samples (20-fold dilution of reaction mixture) were subjected to SPR measurement. Flow rate = 20 μL/min, temp = 25 °C.

Table 1
Kinetic constants of EGFR and MAM to lyso-GM3/lyso-GM3 mimetic estimated by SPR

Ligand	Analyte protein	k_{assoc} (M^{-1} s^{-1})	k_{dissoc} (s^{-1})	K_a (M^{-1})	K_d (M)
Lyso-GM3	EGFR	4.41×10^5	0.745	5.92×10^5	1.69×10^{-6}
	MAM	7.66×10^5	0.303	2.53×10^6	3.96×10^{-7}
Lyso-GM3 mimetic	EGFR	8.09×10^5	0.835	9.69×10^5	1.03×10^{-6}
	MAM	3.91×10^6	0.548	7.14×10^6	1.4×10^{-7}

4). Binding affinities of EGFR to immobilized lyso-GM3 or lyso-GM3 mimetic were in a similar range as the binding affinity of the lectin MAM (which recognizes NeuAcα2-3 Gal) to immobilized lyso-GM3 or its mimetic.

These observations clearly demonstrated the direct interaction of EGFR to the lyso-GM3 immobilized sensor chip via an amide bond. The interaction demonstrated here cannot be perfectly assumed to be the phenomenon between EGFR and lyso-GM3 because free amino moiety of lyso-GM3 was obliterated. However, the adsorption of EGFR on the immobilized lyso-GM3 indicates that the specific interaction can occur without a free amino group. Moreover, the immobilized lyso-GM3 mimetic, as well as the immobilized lyso-GM3, adsorbed EGFR. The result indicates that the sugar part of ligands may play an important role on the interaction with EGFR, whereas GM3/lyso-GM3 or its mimetics show different biological/physical properties.

Two mechanisms of interaction between EGFR and the carbohydrate moiety of GM3 should be considered: (i) GM3 may bind to the protein moiety of EGFR through carbohydrate-to-protein interactions (CPI), based on the provision that EGFR protein *per se* has GM3-binding property; and (ii) GM3 may bind to the N-linked glycan of EGFR through carbohydrate-to-carbohydrate interactions (CCI). The method based on SPR as described above cannot distinguish between (i) and (ii). The binding affinity of CCI determined by SPR was similar to that of CPI ($K_a \sim 10^{-8}$ M), but the rapidity of the binding of CCI is higher than that of CPI [for review see 41]. So we treated EGFR with PNGase F and obtained an N-deglycosylated product (Fig. 5A). However, the effect of treatment with PNGase on the glycolipid-immobilized surface could not be observed by SPR. Further studies using SPR are required to understand the detailed interactions between EGFR and glycolipids.

4. Experimental

4.1. General methods and chemicals

^{1}H and ^{13}C NMR spectra were recorded on a 600-MHz JEOL ECP-600 spectrometer, and peaks were assigned using COSY and HMQC techniques. All reactions were monitored by thin-layer chromatography (TLC) on Silica Gel 60F_{254} (E. Merck), detected by UV light or visualized by spraying with anisaldehyde–H_2SO_4 or phosphomolybdic acid reagent. Column chromatography was performed on Silica Gel 60 (70–230 mesh, E. Merck) or flash Silica Gel. ESIMS spectra were recorded on a Bruker HCT ultra-12S Mass Spectrometer.

Sodium methoxide, sodium acetate, polyoxyethylene (20) sorbitan monolaurate (Tween-20), polyoxyethylene (9) octylphenyl ether (NP-40) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and lyso-GM3 was from Takara Bio Inc. (Shiga, Japan). Epidermal growth factor receptor (human) was obtained from Sigma–Aldrich (Milwaukee, WI). The amine coupling kit and research grade CM5 sensor chips were from Biacore. MAM (*Maackia amurensis* lectin) was purchased from Seikagaku Corporation (Tokyo, Japan). N-Glycosidase F (PNGase F) was from Roche. Bio-Safe Coomassie Stain was from Bio-Rad.

4.2. Synthesis of lyso-GM3 mimetic

Fully O-acetylated lyso-GM3 mimetic (compound **1**; 2-aminododecyl (5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosyl)onate-(2→3)-2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside) was prepared as described previously.[7]

Compound **1** (48.7 mg, 39.4 μmol) was dissolved in dry MeOH (3 mL), and 1 mg of NaOMe was added to the solution. The solution was stirred for 15 h at room temperature, and then neutralized with cation-exchange resin (Amberlite IR120B NA, H^+ form). After filtration, the filtrate was evaporated, and lyophilized to give **2** as a white solid: 30.4 mg, 94.4%. ^{1}H NMR (600 MHz, methanol-d_4): δ 7.70 (m, 2H, –NHCO–), 4.43 (dd, 1H, $J_{1',2'}$ 7.7 Hz, H-1′), 4.30 (dd, $J_{1,2}$ 8.2 Hz, 1H, H-1), 2.85 (dd, 1H, $J_{3ax'',3eq''}$ 12 Hz, $J_{3eq'',4''}$ 3.8 Hz, H-3eq″), 2.01 (s, 3H, –NHAc), 1.73 (m, 1H, H-3ax″), 1.41–1.29 (m, –CH_2–), 0.89 (t, 3H, *J* 7.2 Hz, –CH_3). ^{13}C NMR: δ 174.16 (1C, –COOH), 173.60 (1C, –NHCOCH_3), 160.16 (1C, –C–NH_2), 103.77 (1C, C-1′), 102.82 (1C, C-1), 99.77 (1C, C-2″), 79.57 (1C, C-4), 76.27, 75.73, 75.16, 74.79, 74.41, 73.60, 73.51, 71.65, 69.48, 68.76, 68.02,

67.64, 63.20 (1C, C-9″), 61.40 (1C, C-6′), 60.57 (1C, C-6), 52.63 (1C, C-5″), 50.93, 40.81 (1C, C-3″), 33.40, 33.06, 31.75, 29.58, 29.42, 29.35, 29.14, 25.80, 25.74, 22.41, 21.27 (1C, –$NHCOCH_3$), 13.11 (1C, –CH_3). ESIMS (positive-ion): calcd for $C_{35}H_{64}N_2O_{19}Na$ $[M+Na]^+$, 839.40; found $[M+Na]^+$, 839.4. (Negative-ion): calcd for $C_{35}H_{63}N_2O_{19}$ $[M-H]^-$, 815.41; found $[M-H]^-$, 815.4.

4.3. SPR measurements

Surface plasmon resonance (SPR) experiments were performed with a BIACORE X instrument. All experiments were performed in degassed phosphate-buffered saline (PBS) (pH 7.4) containing 0.005% Tween-20, at 25 °C.

Lyso-GM3 and its mimetic (compound **2**) were immobilized on a Biacore CM5 sensor chip by amine coupling as follows: CM-dextran matrix was activated with 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) at 5 μL/min for 7 min. The ligand (100 μM) in 10 mM NaOAc buffer (pH 6.0) was then allowed to flow over the activated surface at the same flow rate for 7 min, and the remaining reactive sites were blocked with 1.0 M ethanolamine·HCl at the same flow rate for 7 min. Immobilized lyso-GM3 (Fig. 1A) and lyso-GM3 mimetic (compound **2**, Fig. 1B) gave signals of 175 and 170 resonance units (RU), respectively. The running buffer for the immobilizing reactions was PBS at pH 7.4, run at a flow rate of 10 μL/min at 25 °C.

Analyte proteins at various concentrations were allowed to flow over the glycolipid-immobilized surface at 20 μL/min, and association/dissociation constants were monitored. Bovine serum albumin (BSA) was used as a control, nonspecific protein.

After each analysis, the surface was washed with regeneration buffer (0.05% NP-40 in PBS) and equilibrated with running buffer.

4.4. N-Deglycosylation of EGFR

An aliquot of EGFR (1.3 μg of protein) was dissolved in 50 μL of 200 mM sodium phosphate buffer (pH 7.6), containing 0.2% β-mercaptoethanol and 0.5% NP-40. The mixture was incubated with 5 U of PNGase F for 24 h at 37 °C. As a control, the same amount of EGFR was incubated under the same conditions without PNGase F. The incubation mixture was subjected to 7.5% SDS–PAGE and stained by Bio-Safe Coomassie Stain.

Acknowledgments

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