Ganglioside Microarrays for Toxin Detection†

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Microarrays of lipids containing gangliosides were fabricated on surfaces coated with γ-aminopropylsilane. A fluorescence microarray scanner was used to detect the binding of toxins to these arrays. The specific binding of cholera and tetanus toxins to microspots containing GM1 and GT1b gangliosides, respectively, was demonstrated. These results suggest the possibility of using ganglioside microarrays for multiplexed toxin detection and the screening of compounds that can inhibit toxin binding.

Introduction

This paper describes the fabrication of lipid microarrays containing gangliosides on surfaces coated with γ-aminopropylsilane (GAPS) and their use for detecting the binding of toxins by fluorescence imaging.

Microarrays consist of spatially indexed microspots ("probe" microspots) comprising immobilized molecules of biological interest. When exposed to a sample of interest (the "target"), binding of molecules in the sample to the microspots occurs to an extent determined by the concentration of that molecule and its affinity for a particular probe microspot. In principle, if the target concentrations are known, the affinity of the target for the different probe microspots can be estimated simultaneously. Conversely, in principle, given the known affinities of the different molecules in the target for each probe microspot, the amounts of binding observed at each microspot may be used to simultaneously estimate the concentrations of multiple analytes in the sample. The attractiveness of microarray technology lies in the ability to obtain highly multiplexed information using small amounts of sample. DNA microarrays[1,2] have revolutionized studies of gene expression and polymorphism analysis, and there is currently tremendous activity in the field of protein microarrays.[3−8]

There is yet another distinct kind of microarray, a membrane microarray,[9−14] which requires unique considerations for fabrication but offers exciting possibilities for biopharmaceutical research. Membrane microarrays are fundamentally different from DNA or conventional protein microarrays; they require the immobilization of the probe molecules of interest and the associated lipids. Another unique aspect of membrane microarrays is the need to keep the probe confined to the microspot while maintaining the desired lateral movement of individual molecules within the microspot—properties that are contradictory and preclude covalent immobilization of the membrane.[9,15,16] Although the self-assembly of lipids limits lateral expansion beyond the printed area, the mechanical stability of noncovalently adsorbed lipids is a very significant issue and can severely compromise the feasibility of membrane microarrays. Supported membranes are particularly susceptible to desorption when withdrawn through air−water interfaces. Boxer and co-workers have reported that supported lipids on bare-glass substrates spontaneously desorb when withdrawn through air−water interfaces.[17] Assays for microarrays require incubation with different reagents and buffers and withdrawal through air−water interfaces between these multiple steps; moreover, conventional microarray scanners are not well suited to scanning slides that are wet. Given these considerations, an ideal surface for membrane microarrays should have properties such that (i) supported membranes on the surface resist physical desorption and (ii) supported membranes on the surface exhibit long-range lateral fluidity. To test the stability of membrane microarrays, we subjected slides with printed membrane microspots (doped with fluorescently labeled lipids) to repeated immersions into buffer and withdrawal through the buffer−air interface and examined the slides by fluorescence microscopy. The lateral fluidity of supported lipids was tested by traditional fluorescence recovery after photobleaching experiments.[12] Screening of several sur-

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(1) Chee, M.; Yang, R.; Hubbell, E.; Berno, A.; Huang, X.; Stern, D.; Winkler, J.; Lockhart, D.; Morris, M.; Fodor, S. *Science* **1996**, *274*, 610−614.

(2) Brown, P. O.; Botstein, D. *Nat. Genet.* **1999**, *21*, 33−37.

(3) de Wildt, R.; Mundy, C.; Gorick, B.; Tomlinson, I. *Nat. Biotechnol.* **2000**, *18*, 989−994.

(4) MacBeath, G.; Schreiber, S. L. *Science* **2000**, *289*, 1760−1761.

(5) Zhu, H.; Klemic, J. F.; Chang, S.; Bertone, P.; Casamayor, A.; Klemic, K. G.; Smith, D.; Gerstein, M.; Reed, M. A.; Snyder, M. *Nat. Genet.* **2000**, *26*, 283−289.

(6) Zhu, H.; Bilgin, M.; Bangham, R.; Hall, D.; Casamayor, A.; Bertone, P.; Lan, N.; Jansen, R.; Bidlingmaier, S.; Houfek, T.; Mitchell, T.; Miller, P.; Dean, R. A.; Gerstein, M.; Snyder, M. *Science* **2001**, *293*, 2101−2105.

(7) Zhu, H.; Snyder, M. *Curr. Opin. Chem. Biol.* **2001**, *5*, 40−45.

(8) Kodadek, T. *Chem. Biol.* **2001**, *8*, 105−115.

(9) Lahiri, J.; Kalal, P.; Frutos, A. G.; Jonas, S. J.; Schaeffler, R. *Langmuir* **2000**, *16*, 7805−7810.

(10) Lahiri, J.; Jonas, S. J.; Frutos, A. G.; Kalal, P.; Fang, Y. *Biomed. Microdevices* **2001**, *3*, 157−164.

(11) (a) Fang, Y.; Frutos, A. G.; Lahiri, J. *J. Am. Chem. Soc.* **2002**, *124*, 2394−2395. (b) Fang, Y.; Frutos, A. G.; Lahiri, J. *ChemBioChem* **2002**, *3*, 987−991.

(12) Groves, J. T.; Ulman, N.; Boxer, S. G. *Science* **1997**, *275*, 651−653.

(13) Hovis, J.; Boxer, S. G. *Langmuir* **2000**, *16*, 894−897.

(14) Bieri, C.; Ernst, O. P.; Heyse, S.; Hofmann, K. P.; Vogel, H. *Nat. Biotechnol.* **1999**, *17*, 1105−1108.

(15) Covalent immobilization of the entire supported membrane is not desirable; surfaces with covalently immobilized amphiphilic tethers have been shown to bind lipids, resulting in the formation of supported lipid bilayers.

(16) Raguse, B.; Braach-Maksvytis, V.; Cornell, B. A.; King, L. G.; Osman, P. D. J.; Pace, R. J.; Wieczorek, L. *Langmuir* **1998**, *14*, 648−659.

(17) Cremer, P. S.; Boxer, S. G. *J. Phys. Chem. B* **1999**, *103*, 2554−2559.

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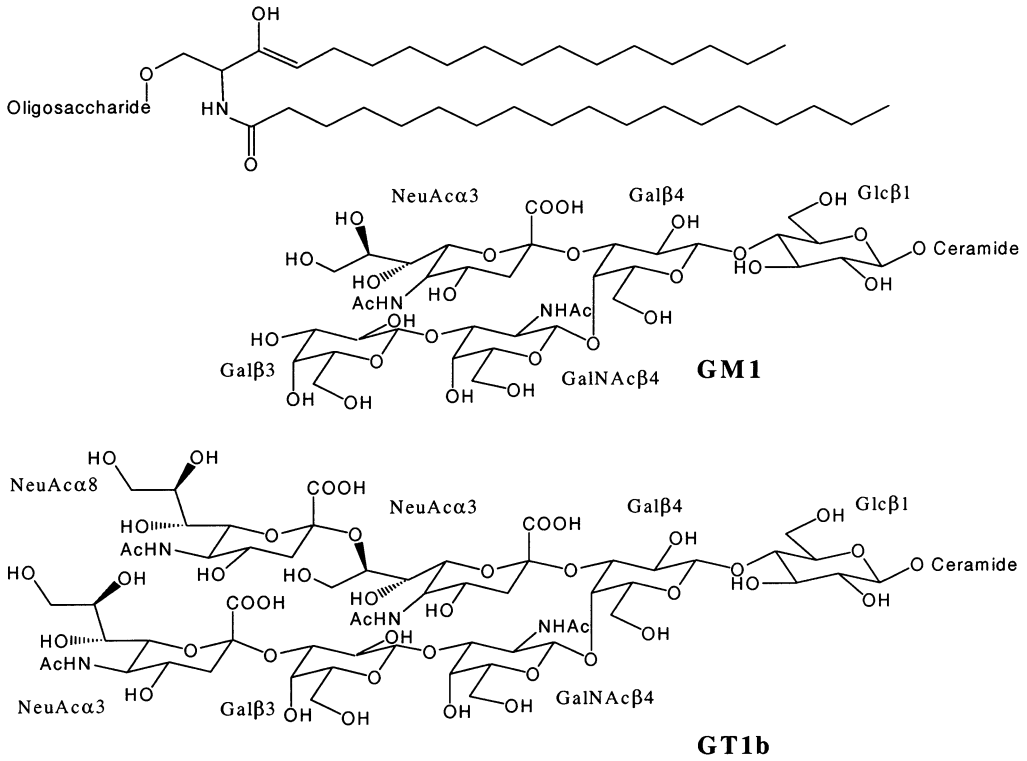

Figure 1. Structures of the GM1 and GT1b gangliosides.

faces revealed that those derivatized with GAPS provided the best balance of these properties: microarrays on GAPS resisted desorption (independent of the phase of the lipids), and supported membranes on GAPS exhibited lateral fluidity (with a mobile fraction of ~0.5).[11] Furthermore, microarrays of G protein-coupled receptors on GAPS were shown to bind ligands with affinities and specificities consistent with the literature, which demonstrated the feasibility of fabricating membrane protein microarrays.[11]

A particularly significant and topical application of membrane microarrays is the detection of bacterial toxins and the testing of compounds as potential inhibitors. Most bacterial toxins consist of two "domains": the first is involved in binding to the cell membrane (binding domain, termed B) and the second is involved in intracellular enzymatic activity (activating domain, termed A). Various molecules on the surface of the host cell are targets for toxin binding.[18] For example, cholesterol is the target molecule for bacteria from the genera *Streptococcus*, *Bacillus*, *Clostridium*, and *Listeria*. Some toxins bind to proteins on the cell surface during pathogenesis; examples include the binding of the diphtheria toxin (*Corynebacterium diphtheriae*)[19] and the anthrax toxin (*Bacillus anthracis*).[18] A large number of bacterial toxins target carbohydrate-derivatized lipids on the cell surface, often with high specificity. These lipids, glycosylated derivatives of ceramides referred to as sphingoglycolipids, can be classified into cerebrocides (ceramide monosaccharide), sulfatides (ceramide monosaccharide sulfates), and gangliosides (ceramide oligosaccharides).[20] Gangliosides comprise 5−10% of the lipid composition of the plasma membrane of neuronal and glial cells.[21] One of the best-studied examples of toxin−ganglioside interactions is the binding of the (cholera) toxin produced by *Vibrio cholerae* to the ganglioside GM1. The GM1 ganglioside contains a pentasaccharide (Figure 1) consisting of Gal(β1-3)GalNAc-(β1-4)(NeuAc(α2-3))Gal(β1-4)Glc(β1-1)ceramide. Studies suggest that the binding epitope of GM1 for the cholera toxin includes the internal Gal and almost all of the external Galβ3, NeuAcα3, and possibly the methyl moiety of the acetamido group of GalNAcβ4.[22] The specificity of toxin−carbohydrate interactions is well demonstrated by differences in the binding epitopes between the tetanus and cholera toxins. The tetanus toxin (produced by *Clostridium tetani*) binds specifically to the ganglioside GT1b; this ganglioside (NeuAc(α2-3)Gal(β1-3)GalNac(β1-4)(NeuAc(α2-8)NeuAc(α2-3))Gal(β1-4)Glc(β1-1)ceramide) (Figure 1) contains two sialic acid (NeuAcα3 and NeuAcα8) residues appended to the GM1 ganglioside. Binding studies have shown that the Galβ3GalNAcβ4-NeuAcα8NeuAcα3Galβ4 moiety is involved in binding to the Hc fragment of the tetanus toxin.[23] The Hc fragment is involved in interactions with the terminal NeuAcα8 residue; the lack of this sugar in the GM1 ganglioside explains the reduced affinity of the tetanus toxin for GM1. Conversely, extensions to the terminal Galβ3 residue of GM1 are not favorable for binding to the cholera toxin; therefore, the additional sialic acid (NeuAcα3) in GT1b results in poor binding of the cholera toxin to the GT1b ganglioside.

The presentation of the carbohydrates themselves has a profound influence on various biological processes such as pathogenesis and cell adhesion. The high affinity and specificity of carbohydrate-mediated recognition are achieved through simultaneous interactions with multiple

(18) Rappuoli, R.; Montecucco, C. *Guidebook to Protein Toxins and Their Use in Cell Biology*; Oxford University Press: Oxford, 1997.
(19) Brooke, J. S.; Cha, J.-H.; Eidels, L. *Biophys. Biochem. Res. Commun.* **1998**, *248*, 297−302.
(20) Voet, D.; Voet, J. G. *Biochemistry*; John Wiley and Sons: New York, 1995.
(21) Derry, D. M.; Wolfe, L. S. *Science* **1967**, *158*, 1430−1432.
(22) Ångstrom, J.; Teneberg, S.; Karlsson, K.-A. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 11859−11863.
(23) Fotinou, C.; Emsley, P.; Black, I.; Ando, H.; Ishida, H.; Kiso, M.; Sinha, K. A.; Fairweather, N. F.; Isaacs, N. W. *J. Biol. Chem.* **2001**, *276*, 32274−32281.

carbohydrate moieties.[24] Carbohydrate ligands are typically presented in clusters on the cell surface, which enables the simultaneous binding of proteins to multiple ligands. The binding domain of the cholera toxin itself consists of a pentamer of B subunits; multivalent interactions with several GM1 groups lead to enhanced affinity of the toxin for the cell surface.[24] Non-cell-based methods for studying carbohydrate recognition must take into account the ligand density that simulates the actual presentation of the ligand on the cell. Kahne and coworkers have demonstrated that solution affinities of monovalent ligands do not necessarily correlate with polyvalent avidities.[25] Mrksich and others have used model surfaces based on self-assembled monolayers presenting defined mole fractions of carbohydrate ligands covalently attached to the surface.[26] Although these well-defined surfaces are excellent for studying biomolecular recognition, a significant limitation is the lack of lateral fluidity in these systems. Supported membranes enable the biomimetic presentation of carbohydrate ligands enabling processes such as protein (or cholesterol) mediated clustering of the ligands.[27] The development of lipid microarrays for studying carbohydrate-mediated recognition combines the convenience and throughput provided by microarrays with the near-native environment provided by supported lipids.

In this paper, we describe the fabrication of microarrays of lipids containing the GM1 and GT1b gangliosides and demonstrate their use for detecting toxins and screening of compounds as potential inhibitors.

Experimental Section

Materials. 1,2-Dilauroyl-*sn*-glycero-3-phosphocholine (DLPC) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL). Monosialoganglioside (GM1), trisialoganglioside (GT1b), bungarotoxin, cholera toxin B subunit (CTx), and FITC-labeled cholera toxin B subunit (FITC−CTx) were obtained from Sigma Chemical (St. Louis, MO). Tetanus toxin fragment C (TTx) and FITC-labeled tetanus toxin fragment C (FITC−TTx) were from Calbiochem (Pasadena, CA). All toxins were handled with extreme care; a 2 M sodium hydroxide solution or bleach was used for decontamination. All other chemicals were obtained from Sigma.

Array Fabrication and Binding Assays. The fabrication of lipid microarrays was carried out using a quill-pin printer (Cartesian Technologies model PS 5000) equipped with software for programmable aspiration and dispensing. Before printing, DLPC (1 mg/mL) in the absence and presence of 4 mol % ganglioside (GM1 or GT1b) in 20 mM phosphate buffer (pH 7.4) was sonicated to clarity. For printing, 25 μL of each lipid solution was added to different wells of a 384 well microplate. Replicate microspots were obtained using a single insertion of the pin into the solution. To prevent contamination due to carryover between different lipid solutions, an automatic wash cycle was incorporated that consisted of consecutive washes of the pin in ethanol and water. After printing, the arrays were incubated in a humid chamber at room temperature for 1 h and then used for toxin binding experiments. For the binding assays, each individual array was incubated with 20 μL of a solution containing labeled toxin (0.031−2 nM) in the absence and presence of varying amounts (0−100 nM) of unlabeled toxin. The binding buffer used for all experiments was 20 mM phosphate buffer (pH 7.4, 0.2% BSA). Arrays were imaged using a GenePix 4000B fluorescence scanner.

Results and Discussion

Fabrication of Microarrays. Vesicles containing gangliosides were obtained by sonicating thin films of DLPC and gangliosides (4% GM1 or GT1b) in buffer. We used DLPC as a host lipid because the lipid is in the fluid phase at room temperature and is not prone to chemical degradation like egg phosphatidylcholine. The printing of arrays was carried out by contact pin printing on GAPS-coated slides. The incubation of slides in a humid chamber after printing was carried out to enable a steady-state lateral redistribution of the lipid molecules in the supported membrane. Gangliosides tend to form aggregates[28] that form moving rafts in the cell membrane;[28] in artificially reconstituted membranes, the phase separation behavior of gangliosides depends on the oligosaccharide, on the presence of cholesterol, and on differences between the hydrocarbon moieties of the ganglioside and the phospholipid.[29,30]

Toxin Binding Assays. The binding assays were carried out by incubating arrays of the gangliosides with the appropriate solution, washing with buffer to remove unbound toxins (and potential inhibitors), and scanning using a fluorescence scanner (see Experimental Section for details). The binding assays were designed to test (I) the selectivity of binding, (II) the specificity of inhibition, and (III) the dose dependency of binding and inhibition.

(I) Selectivity of Binding. Microarrays are naturally suited for simultaneously screening the binding of a compound to multiple probes. In a typical primary screening experiment, the array is incubated with a compound at a particular concentration; if a positive signal (a "hit") is obtained for a probe microspot, more detailed analysis (e.g., dose dependency studies described below) is carried out. Our studies were aimed at simply establishing the specificity of binding to ganglioside microarrays using known toxin−ganglioside interactions.

Figure 2A−C shows fluorescence false color images of three identical microarrays on a single GAPS slide; each microarray consists of three replicate microspots of DLPC (top row), DLPC doped with GM1 (middle row), and DLPC doped with GT1b (bottom row). The first microarray was treated with buffer only and serves as a negative control. As expected, no signal is observed on any of the microspots (Figure 2A). The second microarray (Figure 2B) was incubated with a solution of fluorescently labeled cholera toxin (B subunit, FITC−CTx). Strong binding to microspots containing the GM1 ganglioside is observed, as expected, although weak binding (<10% of the signal obtained with GM1) to microspots of DLPC and DLPC doped with GT1b is also observed. When the microarray was treated with a solution containing fluorescently labeled tetanus toxin (C fragment, FITC−TTx) (Figure 2C), the highest amount of binding was found to correspond to microspots containing the GT1b ganglioside, in accordance with the known specificity of the toxin. The binding signal is lower than that observed for binding of FITC−CTx to GT1b; suboptimal amounts of GT1b in the mixed lipid or issues with labeling of the tetanus toxin could be possible reasons for the poorer signal. We also observe binding of FITC−TTx to the GM1 microspots; the signal is approximately 35% of that observed for binding to GT1b microspots (Figure 2D). Previous researchers have reported that the tetanus toxin does not bind to GM1; we

(24) Mammen, M.; Seok-Ki, C.; Whitesides, G. M. *Angew. Chem., Int. Ed.* **1998**, *37*, 2755−2794.
(25) Liang, R.; Loebach, J.; Horan, N.; Ge, M.; Thompson, C.; Yan, L.; Kahne, D. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 10554−10559.
(26) Mrksich, M. *Chem. Soc. Rev.* **2000**, *29*, 267−273.
(27) Song, X.; Shi, J.; Swanson, B. *Anal. Biochem.* **2000**, *284*, 35−41.
(28) Simons, K.; Ikonen, E. *Nature* **1997**, *387*, 569−572.
(29) Radhakrishnan, A.; Anderson, T. G.; McConnell, H. M. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 12422−12427.
(30) Masserini, M.; Palestini, P.; Freire, E. *Biochemistry* **1989**, *28*, 5029−5034.

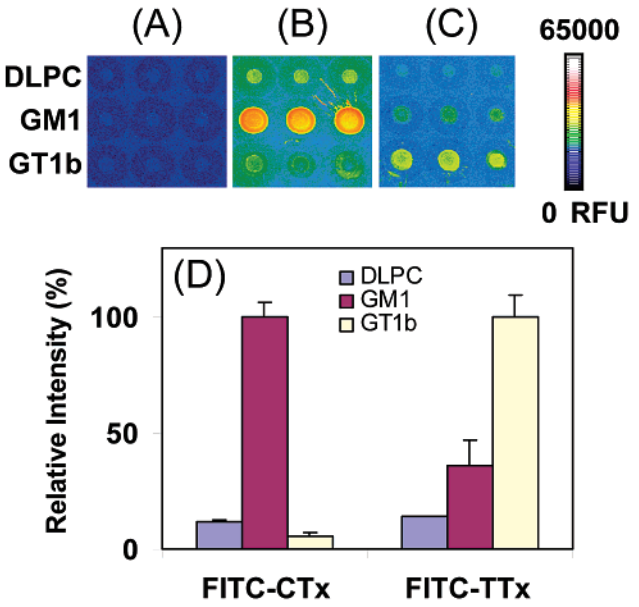

Figure 2. Fluorescence images of microarrays consisting of DLPC (top row), DLPC doped with 4 mol % GM1 (middle row), and DLPC doped with 4 mol % GT1b (bottom row), treated with solutions of toxins. The images correspond to microarrays treated with (A) buffer only, (B) 1 nM fluorescein-labeled cholera toxin (B subunit, FITC−CTx), and (C) 2 nM fluorescein-labeled tetanus toxin (C fragment, FITC−TTx). (D) Histograms showing the relative amounts of binding of the labeled cholera and tetanus toxins to the ganglioside microarrays. The data are normalized to the binding signal of FITC−CTx to GM1 and FITC−TTx to GT1b. RFU = relative fluorescence units.

hypothesize that this binding is nonspecific in nature and appears accentuated due to the inherently low net signal (signal minus background) (∼3000 relative fluorescence units (RFU)) for binding of FITC−TTx to GT1b.

(II) Selectivity of Inhibition. Since labeling of molecules with fluorescent dyes is tricky, compounds are more commonly screened using competition assays in which the unlabeled compound is allowed to compete for probe binding sites with a cognate, labeled ligand for that probe. The decrease in fluorescence relative to an array incubated only with the labeled ligand provides a measure of inhibition by the unlabeled compound. In a typical initial screen, the unlabeled compound is present in large excess (e.g., 100-fold) and a significant (e.g., >50%) decrease in fluorescence leads to further investigation of that compound.

Figure 3 shows fluorescence images of four microarrays (identical in probe content to that used in Figure 2) that were treated with solutions containing FITC−CTx and either the tetanus toxin, bungarotoxin, or unlabeled cholera toxin. Figure 3A (identical to the array in Figure 2B), in which the array was treated with fluorescently labeled cholera toxin, provides the reference for measuring inhibition. Figure 3B is a fluorescence image of an array treated with a mixture of the labeled cholera toxin (1 nM) and unlabeled tetanus toxin (100 nM). There is a small but insignificant decrease in the amount of binding to the GM1 ganglioside microspots; interestingly, the small amount of binding of FITC−CTx to microspots of the GT1b toxin (see Figure 2B) is completely inhibited. The presence of bungarotoxin also causes no significant decrease in the amount of binding of FITC−CTx (Figure 3C). When the microarray was incubated with a mixture containing excess unlabeled cholera toxin (100 nM), there was essentially complete inhibition of binding of FITC−CTx to the GM1 microspots (Figure 3D). In a corresponding series of experiments, we observed specific inhibition of FITC−TTx binding by the unlabeled tetanus toxin (data not shown). These experiments demonstrate the feasibility of screening potential inhibitors against toxins using ganglioside microarrays.

(III) Dose Dependency of Binding and Inhibition. One of the questions that arises in the development of a microarray-based assay (or any solid-phase assay) is whether the affinity between two compounds changes upon immobilization of one of the binding partners (as a probe). For microarrays, the estimation of binding constants requires direct comparisons of the binding signals between microspots treated with different concentrations of an analyte. This comparison is not straightforward as it often requires measurements of small differences in fluorescence and demands much greater precision than that required for screening applications.

In the microarrays described here, the probe (GM1or GT1b) was mixed with a host lipid (DLPC). It is reasonable to assume that for a particular preparation of the DLPC/ganglioside mixture, the surface mole fraction of ganglioside (and DLPC) is the same from spot to spot. We can estimate the surface binding capacity for the cholera toxin at a ganglioside density of 4 mol %; this estimation (outlined below) does not take into account inhomogeneities in the distribution of gangliosides in the host lipid. The radius of the pentameric binding domain of the cholera toxin is ∼3 nm;[31] assuming a close packed structure, the number of cholera toxin molecules per square micron is $\sim 2.3 \times 10^4$. Assuming 1:1 binding between GM1 and each subunit of the cholera toxin, the number of binding sites corresponding to a monolayer of the cholera toxin is $\sim 1.2 \times 10^5/\mu m^2$. Using Langmuir−Blodgett techniques, the area occupied by a GM1 molecule in a GM1 monolayer at the air−water interface was estimated to be ∼0.6 nm^2.[32] Assuming an area of ∼0.5 nm^2 per DLPC molecule, we estimate that there are $\sim 2 \times 10^6$ molecules of DLPC and $\sim 8 \times 10^4$ molecules of GM1 per μm^2. Therefore, the number of GM1 molecules (at 4 mol %) per unit area is approximately half the number of binding sites required for binding a full monolayer of the cholera toxin. In reality, given the nanomolar affinity of the cholera toxin for ganglioside-presenting surfaces,[33,34] binding of the toxin is essentially irreversible.[24,35,36] Therefore, assuming a random sequential adsorption model in which approximately 55% coverage is maximally possible,[37,38] the number of cholera toxin molecules that can bind to a surface presenting GM1 molecules is $\sim 6.3 \times 10^4$ molecules per μm^2. Therefore, a DLPC surface doped with ∼4 mol % GM1 is probably sufficient for supporting the maximum possible amount of binding of the cholera toxin.

Figure 4 shows fluorescence images of microarrays of the GT1b ganglioside (4 mol %) treated with different concentrations of FITC−CTx. The dependency of the signal

(31) Kuziemko, G. M.; Stroh, M.; Stevens, R. C. *Biochemistry* **1996**, *35*, 6375−6384.
(32) Luckham, P.; Wood, J.; Froggatt, S.; Swart, R. *J. Colloid Interface Sci.* **1993**, *156*, 164−172.
(33) MacKenzie, C. R.; Hirama, T.; Lee, K. K.; Altman, E.; Young, N. M. *J. Biol. Chem.* **1997**, *272*, 5533−5538.
(34) Lauer, S.; Goldstein, B.; Nolan, R. L.; Nolan, J. P. *Biochemistry* **2002**, *41*, 1742−1751.
(35) High-affinity binding at surfaces often appears irreversible because of mass transport limited rebinding. This apparent irreversibility can be reversed by washing the chip with solutions containing the probe or other molecules that bind to the sample.
(36) Rao, J.; Yan, L.; Xu, B.; Whitesides, G. M. *J. Am. Chem. Soc.* **1999**, *121*, 2629−2630.
(37) Higher coverages can be obtained depending on the amount of equilibration.
(38) Meineke, M. A.; Gezelter, J. D. *J. Chem. Phys. B* **2001**, *105*, 6515−6519.

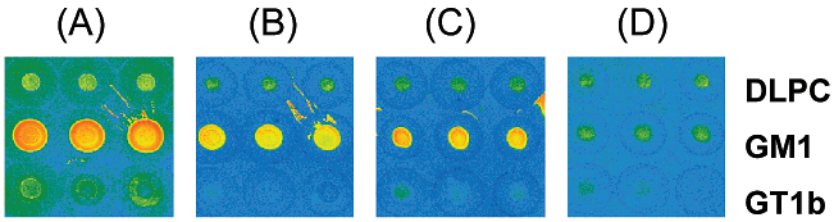

Figure 3. Fluorescence images of microarrays of DLPC (top row), DLPC doped with 4 mol % GM1 (middle row), and DLPC doped with 4 mol % GT1b (bottom row), treated with solutions containing FITC−CTx in the absence and presence of different inhibitors. (A) Image of a microarray treated with FITC−CTx (1 nM). (B) Image of a microarray treated with FITC−CTx (1 nM) and unlabeled tetanus toxin (100 nM). (C) Image of a microarray treated with FITC−CTx (1 nM) and unlabeled bungarotoxin (100 nM). (D) Image of a microarray treated with FITC−CTx (1 nM) and unlabeled cholera toxin (100 nM).

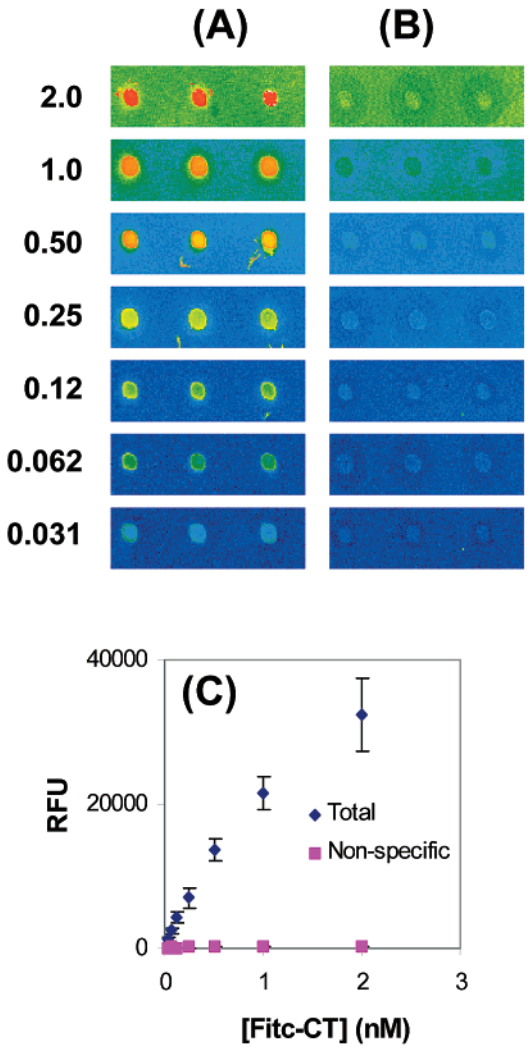

Figure 4. (A) Fluorescence images of microarrays of the GM1 ganglioside (4 mol % in DLPC) treated with solutions of FITC−CTx at concentrations ranging from 0.031 to 2.0 nM. (B) Fluorescence images of an identical set of microarrays treated with FITC−CTx at the same concentrations and excess unlabeled cholera toxin (100 nM). These residual signals provide an estimate of the amount of nonspecific binding at each concentration of FITC−CTx. (C) Graphs showing the concentration dependence of the total fluorescence signal (in RFU) and the signal due to nonspecific binding.

on the concentration of FITC−CTx is obvious. To estimate the amount of nonspecific binding, a corresponding set of microarrays was treated with a mixture containing FITC−CTx at the same concentrations and excess unlabeled cholera toxin. The difference between the signals at each concentration of FITC−CTx provides a measure of the amount of specific binding to the GM1 microspots. Specific

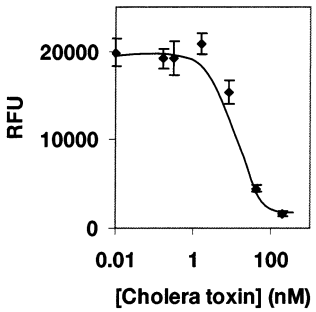

Figure 5. Dose-dependent inhibition of the binding of labeled cholera toxin to GM1 ganglioside microarrays by unlabeled cholera toxin. The arrays were treated with solutions containing FITC−CTx (1 nM) and unlabeled cholera toxin at different concentrations (0−200 nM).

binding is observed even at a concentration of ∼30 pM, which demonstrates the excellent sensitivity of the fluorescence assay. The amount of binding is linear at concentrations less than 1 nM. Unfortunately, the background signals at higher concentrations of FITC−CTx are too high to observe saturation of the binding signal.

The binding of the cholera toxin to gangliosides is multivalent; the binding affinity is dependent on the valency of the interaction, and hence the binding cannot be characterized by a single dissociation constant.[24] Instead, we measure the affinity of the compound in terms of an IC50 value. To estimate this affinity, we carried out a competition assay in which GM1 microarrays were treated with increasing concentrations of unlabeled cholera toxin at a fixed concentration of FITC−CTx (1 nM). From the inhibition profile, we estimate $IC_{50} \sim 20$ nM. The IC50 value can be used to estimate an "average" value for the dissociation constant (K_i); we estimate $K_i \sim 13$ nM, which is ∼25 times greater than the affinity of the toxin for the soluble GM1 pentasaccharide.[34,39] The relative contributions of polyvalency and the presentation of GM1 as a ganglioside (as opposed to a free sugar) are presently unclear. These data demonstrate the feasibility of estimating affinities of potential inhibitors using ganglioside microarrays.

(39) The value of the binding constant represents the "average" affinity of the ganglioside microspots for the cholera toxin. The estimation of K_i from the measured IC50 value is given by

$$K_i = \frac{IC_{50}}{1 + \frac{L}{K_L}}$$

where K_i is the equilibrium dissociation constant for the inhibitor, L is the concentration of FITC−CTx, and K_L is the equilibrium dissociation constant of FITC−CTx. There are significant discrepancies in the literature in the reported values of the binding constant between the cholera toxin and the GM1 ganglioside; a reasonable "consensus" value is ∼2 nM.[33] Since we were unable to directly estimate the value of K_L, we use $K_L = 2$ nM and assume that labeling of the toxin does not influence its binding affinity.

From our experiments, we can infer that the detection limits for the unlabeled toxins are ~10 and 50 nM for the cholera and tetanus toxins, respectively, using competitive binding assays. The detection limits for the labeled toxins are 0.03 and 0.06 nM for FITC-labeled cholera and tetanus toxins, respectively.

Conclusions

Microarrays containing components of the cell membrane provide an attractive platform for efficiently studying fundamental aspects of molecular recognition at the cell surface. These studies are of immediate relevance to diverse applications ranging from drug discovery to the detection of pathogens. Until recently, the development of membrane microarrays was hindered by issues such as the printing of lipids, the need to keep the arrays immersed in water, and the poor mechanical stability of supported membranes. The use of GAPS-coated substrates and conventional robotic printing provides a robust platform for assay development on membrane microarrays. Carbohydrate-derivatized lipids and proteins are a particularly interesting set of cell surface ligands;[40,41] they play important roles in pathogenesis and during an immune response. At a time when bacterial resistance to antibiotics[42] and biowarfare[43] are both significant concerns, the development of multiplexed bioanalytical platforms for studying carbohydrates in near native environments is especially pertinent.

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(40) Evans, S. V.; MacKenzie, C. R. *J. Mol. Recognit.* **1999**, *12*, 155−168.
(41) Sears, P.; Wong, C.-H. *Angew. Chem., Int. Ed.* **1999**, *38*, 2300−2324.
(42) Williams, D. H.; Bardsley, B. *Angew. Chem., Int. Ed.* **1999**, *38*, 1173−1193.
(43) Iqbal, S. S.; Mayo, M. W.; Bruno, J. G.; Bronk, B. V.; Batt, C. A.; Chambers, J. P. *Biosens. Bioelectron.* **2000**, *15*, 549−578.