Anal. Chem. **2000,** *72,* 6019−6024

Gangliosides as Receptors for Biological Toxins: Development of Sensitive Fluoroimmunoassays Using Ganglioside-Bearing Liposomes

Anup K. Singh,* Suzanne H. Harrison, and Joseph S. Schoeniger

Chemical & Radiation Detection Laboratory, Sandia National Laboratories, Livermore, California 94550

Gangliosides, glycosphingolipids present in the membranes of neuronal and other cells, are natural receptors for a number of bacterial toxins and viruses whose sensitive detection is of interest in clinical medicine as well as in biological warfare or terrorism incidents. Liposomes containing gangliosides mimic cells that are invaded by bacterial toxins and can be used as sensitive probes for detecting these toxins. We discuss detection of three bacterial toxins—tetanus, botulinum, and cholera toxins using ganglioside-bearing liposomes. Tetanus and botulinum toxins selectively bind gangliosides of the G1b series, namely, GT1b, GD1b, and GQ1b; and cholera toxin binds GM1 very specifically. Unilamellar liposomes containing GT1b or GM1 as one of the constituent lipids were prepared by extrusion through polycarbonate membranes. To impart signal generation capability to these liposomes, fluorophore-labeled lipids were incorporated in the bilayer of liposomes. The fluorescent liposomes, containing both a marker (rhodamine) and a receptor (GT1b or GM1) in the bilayer, were used in sandwich fluoroimmunoassays for tetanus, botulinum, and cholera toxins and as low as 1 nM of each toxin could be detected. The apparent dissociation constants of liposome−toxin complexes were in 10^{-8} M range, indicating strong binding. This is the first report on detection of tetanus and botulinum toxins based on specific recognition by gangliosides. The fluorescent liposomes are attractive as immunoreagents for another reason as well—they provide enormous signal amplification for each binding event as each liposome contains up to 22 000 rhodamine molecules. The present approach using receptors incorporated in bilayers of liposomes offers a unique solution to employ water-insoluble receptors, such as glycolipids and membrane proteins, for sensitive detection of toxins and other clinically important biomolecules.

Bacterial toxins and viruses are often specifically targeted to certain cell types that exhibit characteristic binding sites or specific receptors on their surfaces. The mechanism of cell intoxication by bacterial protein toxins begins with binding of toxin to a cell surface receptor. After binding to the receptor, part or all of the toxin penetrates and, in some cases, is translocated across the bilayer membrane to the inside of the cell where the toxic effect is manifested.[1] The majority of known receptors for bacterial exotoxins are carbohydrates, in the form of either glycolipids or glycoproteins.[2,3] Carbohydrates have long been known as biological receptors, binding of lectins to carbohydrates being the paradigm for protein−sugar interaction.[4] Gangliosides, a type of glycolipid, have been implicated in many cellular functions including cell−cell recognition, interactions with extracellular proteins, receptor for hormones, receptor for viruses such as influenza and Sendai, and receptors for toxins such as cholera, tetanus, and botulinum.[5−7] Botulinum and tetanus toxins have been determined to bind selectively to gangliosides of the series "G1b", namely, GT1b, GD1b, and GQ1b,[8−10] which are present in neuronal cell membranes. Cholera toxin binds very specifically and strongly to cell surface via ganglioside GM1 found on the surface of enterocytes and many other eukaryotic cell types.[10−12] Figure 1 shows the structures of gangliosides GM1 and GT1b. They are composed of a hydrophobic ceramide backbone and an oligosaccharide polar headgroup containing neutral sugars (glucose, galactose, *N*-acetylgalactosamine) and an anionic sugar sialic acid.[13] Binding of a toxin molecule to ganglioside involves recognition of an internal sequence of sugars on the saccharide chain, the number of sialic acid residues and their relative positions being the predominant factors. This feature ensures the specificity of binding as glycolipids not only need to contain the required sugars, they should be presented in the correct order and conformation. Table 1 presents a list of microbial toxins and their receptors. By no means is this a comprehensive list but rather a few examples to elucidate the universality of toxin−glycolipid binding.

* Corresponding author: (e-mail) aksingh@sandia.gov; (phone) 925-294-1260; (fax) 925-294-3020.

(1) Montecucco, C.; Papini, E.; Schiavo. G. In *Sourcebook of bacterial protein toxins*; Alouf, J. E., Freer; J. H., Eds.; Academic Press: New York, 1991; pp 45−56.
(2) Eidels, L.; Proia, R. L.; Hart, D. A. *Microbiol. Rev.* **1983**, *47*, 596−620.
(3) Karlsson, K.; Angstrom, J.; Teneberg, S. In *Sourcebook of bacterial protein toxins*; Alouf, J. E., Freer; J. H., Eds.: Academic Press: New York, 1991; pp 435−443.
(4) Sharon, N.; Lis, H. *Science* **1972**, *177*, 949−959.
(5) Fishman, P. H.; Brady, R. O. *Science* **1976**, *194*, 906−915.
(6) Hakomori, S. I. *J. Biol. Chem.* **1990**, *265*, 18713−18716.
(7) Fishman, P. H.; Pacuzska, T.; Orlandi, P. A. *Adv. Lipid Res.* **1993**, *25*, 165−187.
(8) van Heyningen, W. E.; Miller, P. A. *J. Gen. Microbiol.* **1961**, *24*, 107−119.
(9) Simpson, L. L.; Rapport, M. M. *J. Neurochem.* **1971**, *18*, 1341−1343.
(10) van Heyningen, W. E. *Nature* **1974**, *249*, 415−417.
(11) van Heyningen, W. E.; Carpenter, C. C.; Pierce, N. F.; Greenough, W. B. *J. Infect. Dis.* **1971**, *124*, 415−418.
(12) King, C. A.; van Heyningen, W. E. *J. Infect. Dis.* **1973**, *127*, 639−647.
(13) Voet, D.; Voet, J. G. *Biochemistry*; Wiley: New York, 1990; pp 271−313.

10.1021/ac000846l CCC: $19.00
Published on Web 11/16/2000

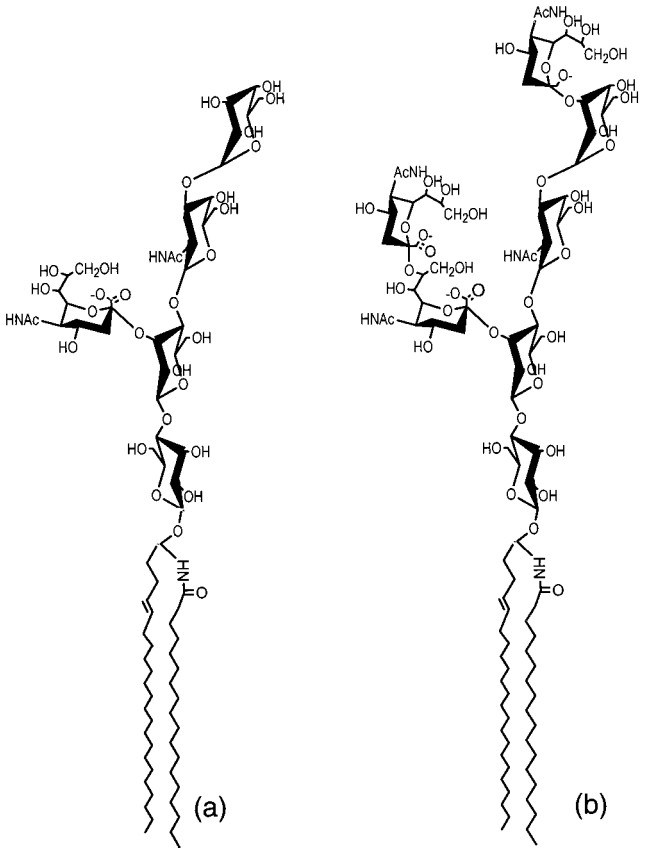

Figure 1. Structure of gangliosides: (a) GM1 (monosialoganglioside); (b) GT1b (trisialoganglioside). GM1 is the specific receptor for cholera toxin, and GT1b is a natural receptor for tetanus and botulinum toxins. GM1 binding to cholera toxin involves hydrogen bonding of terminal sugars in a manner resembling a two-finger pinch, where sialic acid acts as a "thumb" and galactose as a "finger".

Table 1. Lipid Receptors of a Few Bacterial Toxins

source	toxin	receptor lipid
Clostridium tetani	tetanus	GT1b, GD1b, GQ1b
Clostridium botulinum	botulinum	GT1b, GQ1b
Vibrio cholerae	cholera	GM1
Bordetella pertussis	pertussis	GD1a
Shigella dysenteriae	shiga	Gb3
Escherichia coli	heat-labile enterotoxin	GM1
Streptococcus pyogenes	streptolysin O	cholesterol[a]

[a] Sterol.

Gangliosides, despite having specific and relatively strong affinity for their corresponding toxins, have not been widely used as receptors in immunoassays and biosensors. They have generally poor solubility in water owing to their hydrophobic ceramide chain, prohibiting their use as a reagent. Another reason is that, being small molecules, they do not offer many sites for covalent attachment without disrupting their activity. This makes it impractical to conjugate them to a biosensor surface or to label such as enzymes or fluorophores. But, being amphiphilic, they can readily be incorporated in self-assembled lipid structures such as liposomes or planar lipid films. This obviates any need for covalent conjugation and also presents glycolipids in a native environment. Polydiacetylene Langmuir−Blodgett films and liposomes functionalized with sialic acid (a receptor for influenza virus) or GM1 (a receptor for cholera toxin) that undergo color change upon binding of corresponding analytes have been used for biosensing before.[14] Selective multivalent binding of GM1 to cholera toxin has also been exploited to develop sensitive biosensors based on resonance-energy transfer and self-quenching of fluorescence.[15] Although, there has been a number of reports on detection of cholera toxin using GM1, ganglioside−toxin binding has not been extended to detection of other toxins such as tetanus and botulinum toxins.

Botulinum neurotoxin is produced by the anaerobic bacterium *Clostridium botulinum* and is the most toxic substance known: ∼100 billion times more toxic than cyanide and ∼1 million times more poisonous than cobra toxin.[16] It acts on nerve endings to block aceytlcholine release leading to flaccid muscular paralysis. Tetanus toxin, produced by bacterium *Clostridium tetani*, binds to nerve cells, penetrates the cytosol, and blocks neurotransmitter release causing spastic paralysis. Both tetanus and botulinum toxins consist of a single polypeptide chain of ∼150 kDa. Proteolytic cleavage yields two chains, light (50 kDa) and heavy (100 kDa), that are linked by a single disulfide bridge.[17] The C-terminal of the heavy chain, referred to as C fragment, is involved in binding to nerve cells using gangliosides as receptors. Cholera is an enterotoxigenic illness elicited by *Vibrio cholerae* and is characterized by a watery diarrhea leading to life-threatening dehydration and loss of electrolytes. Cholera toxin (CT) is an oligomeric protein of two distinct domains, CT-A (27 kDa) and CT-B (58 kDa). CT-B is responsible for toxin binding to cell surface via ganglioside GM1.[10−12] It is a pentamer of five identical polypeptides, and each unit binds to one GM1 molecule. Hence, CT can form up to a pentavalent bond with a cell surface leading to a very strong interaction.

In this paper, we discuss detection of three bacterial toxins—tetanus, botulinum, and cholera toxins using ganglioside-bearing liposomes. To impart signal-generation capability to these liposomes, rhodamine-labeled lipids were incorporated in their bilayer. An alternate way of incorporating signal molecules is their encapsulation in the aqueous core of a liposome, but their leakage upon storage is a serious concern. The fluorescent liposomes were used in heterogeneous sandwich immunoassays for detection of the bacterial toxins. Although binding of gangliosides to toxins is quite strong, in many cases one ganglioside binds to multiple toxins of the same family. For example, gangliosides of the G1b series bind to tetanus as well as botulinum toxin, and consequently, it is not possible to differentiate between the two toxins based on binding to ganglioside GT1b alone. In the present work, for positive identification of each toxin, we have employed a "sandwich" approach where toxin is first captured by a monoclonal

(14) Charych, D. H.; Nagy, J. O.; Spevak, W.; Bednarski, M. D. *Science* **1993**, *261*, 585−588. (b) Reichert, A.; Nagy, J. O.; Spevak, W.; Charych, D. *J. Am. Chem. Soc.* **1995**, *115*, 1146−1147. (c) Charych, D.; Cheng, Q.; Reichert, A.; Kuziemko, G.; Stroh, M.; Nagy, J. O.; Spevak, W.; Stevens, R. C. *Chem., Biol.* **1996**, *3*, 113−120. (d) Pan, J. J.; Charych, D. *Langmuir* **1997**, *13*, 1365−1367.

(15) Song, X. D.; Nolan, J.; Swanson, B. I. *J. Am. Chem. Soc.* **1988**, *120*, 11514−11515. (b) Song, X. D.; Nolan, J.; Swanson, B. I. *J. Am. Chem. Soc.* **1998**, *120*, 4873−4874. (c) Song, X. D.; Swanson, B. I. *Anal. Chem.* **1999**, *71*, 2097−2107.

(16) Singh, B. R. *J. Toxicol.-Toxin Rev.* **1999**, *18*, vii-x.

(17) Simpson, L. L. In *Botulinum Neurotoxins and Tetanus Toxin*; Simpson, L. L., Ed.: Academic Press: New York, 1989.

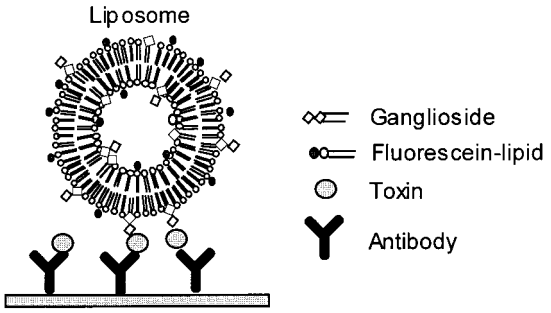

Figure 2. Detection of toxins using fluorescent ganglioside-bearing liposomes. The toxin is first captured using a monoclonal antibody and then exposed to ganglioside-bearing liposomes. Liposomes carry up to 22 000 molecules of rhodamine-labeled lipids, allowing for significant signal amplification for each binding event.

antibody and then detected using ganglioside-bearing fluorescent liposomes as depicted in Figure 2.

Liposomes containing receptors and markers described here offer a number of advantages as immunoreagents. (1) They make it possible for water-insoluble receptors such as glycolipids, sterols, and integral membrane proteins to be used in immunoassays and biosensing. (2) They provide a native environment to these lipophilic receptors thereby maximizing their activity and extending their shelf life. (3) Liposomes have a large surface area and internal volume where thousands of reporter molecules such as fluorescent dyes can be immobilized or entrapped. This leads to large signal amplification for each binding event.[18−20]

EXPERIMENTAL SECTION

Materials. Gangliosides GT1b, GQ1b, and GM1; lipids L-α-distearoylphosphatidylcholine) (DSPC) and L-α-dimyristoylphosphatidylethanolamine) (DMPE); and cholesterol were obtained from Sigma Chemical Co. (St. Louis, MO). Fluorophore-labeled lipids rhodamine-DHPE (*N*-(lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine) were obtained from Molecular Probes, Inc. (Eugene, OR). To avoid the exposure hazard associated with the use of intact toxin, we used commercially available recombinant fragments of toxins (fragments containing ganglioside-binding domain) for this study. The recombinant toxin fragments do not exhibit any native toxicity and, hence, do not pose any health concerns. Recombinant tetanus toxin C fragment and a monoclonal antibody against it were supplied by Boehringer Manheim Corp. (Indianapolis, IN). Cholera toxin B subunit was obtained from Sigma, and the monoclonal antibody against it was purcahsed from BioDesign Laboratories (Kennebunk, ME). Botulinum toxin C fragment and the antibody against it were supplied by Ophidian Pharmaceuticals (Madison, WI). Bovine serum albumin (BSA), casein, and gelatin were obtained from Sigma.

Preparation of GTIb and GM1 Liposomes. Unilamellar liposomes were prepared by extrusion through polycarbonate membranes as described elsewhere.[18] Stock solutions of lipids, gangliosides, and cholesterol were prepared in 2:1 chloroform/methanol (v/v) mixture. A 60-mg sample of lipids was mixed in the mole ratio of 42.5:42.5:10:5 (DSPC/cholesterol/rhodamine-DHPE/GT1b or GM1) in a 25-mL round-bottomed flask. The flask was connected to a rotary evaporator, and the lipid solution was dried thoroughly under vacuum to form a thin lipid film on the inside wall of the flask. Filtered and degassed buffer, either 50 mM citrate buffer at pH 6.0 or phosphate-buffered saline (PBS) at pH 7.4, was added to the dried lipids. The flask was submerged in a water bath at 60 °C and rotated vigorously to form multilamellar vesicles (MLVs). The MLV suspension was sonicated briefly in a bath sonicator to reduce the average size of the liposomes. This solution was then loaded into the syringe of a pneumatic automatic liposome extruder (Liposofast, Avestin, Inc., Vancouver, Canada) and extruded 31 times through two stacked 100-nm polycarbonate filters. The resulting unilamellar liposomes were centrifuged at 3000 rpm for 20 min to remove residual multilamellar vesicles and aggregated lipids. The liposome solution was stored at 4 °C until further use.

Characterization of Liposomes. Hydrodynamic diameters of unilamellar liposomes were estimated by dynamic light scattering (DLS) using a commercial device (Zeta Plus, Brookhaven Instruments Corp.). The liposome suspension was centrifuged at 3000 rpm for 15 min prior to size measurement to remove dust particles and aggregated lipid structures. Samples for size measurement were prepared by adding 25 μL of liposome to 2 mL of 10 mM phosphate (pH 7.2). The solution was filtered using a 0.2-μm syringe filter and dispensed into a clean plastic cuvette. Measurements were performed at a 90° scattering angle using a 633-nm diode laser, and correlation function was generated by a BI-9000AT digital correlator. The data were analyzed using the constrained regularization method,[21] resulting in a size distribution characterized by a mean diameter and variance. The ζ potential of the liposome suspension was measured by electrophoretic light scattering (ZetaPlus, Brookhaven Instruments), which is based on the scattering of light from particles that move in a liquid under the influence of an applied electric field. Liposomes were diluted in a 10 mM KCl solution for the measurement of ζ potential.

Liposome concentration, number of receptors (GT1b) per liposome, and number of rhodamines per liposome were determined as explained elsewhere.[18] Briefly, 25 μL of liposomes was added to a quartz cuvette containing 1.5 mL of methanol and 20 μL of 0.1 N NaOH. The absorbance of this solution was measured at 560 nm to estimate the concentration of rhodamine-DHPE using an extinction coefficient of 95 000 M^{-1} cm^{-1}. Since the mole percentage of rhodamine-DHPE is known, the total lipid concentration can be determined. Knowing the size of a liposome and the projected headgroup areas of the constituent lipids, the number of lipids in spherical unilamellar liposome, N_{tot}, can be estimated as

$$N_{tot} = (\pi/a_L)[d^2 - (d - 2t)^2]$$

where t is the bilayer thickness, d the hydrodynamic diameter, and a_L is the average headgroup area per lipid. The bilayer thickness was assumed to be 40 Å, and a_L was calculated using values of 71, 41, and 19 Å^2 for phosphatidylcholine, phosphatidy-

(18) Singh, A. K.; Kilpatrick, P. K.; Carbonell, R. G. *Biotechnol. Prog.* **1996**, *12*, 272−280.
(19) Singh, A. K.; Carbonell, R. G. In *Nonmedical Applications of Liposomes*; Lasic, D. L., Barenholz, Y., Eds.; CRC Press: Boca Raton, FL, 1995; pp 209−228.
(20) Singh, A. K.; Schoeniger J. S.; Carbonell, R. G. In *Biosensors and Their Applications*; Yang, V. C., Ngo, T. T., Eds.: Kluwer Academic/Plenum Publishimg: New York, 1999; pp 131−145.
(21) Provencher, S. W. *Comput. Phys. Commun.* **1982**, *27*, 213−227.

lamine, and cholesterol, respectively,[22] weighted by the mole fraction of each component. The headgroup area of rhodamine-DHPE was assumed to be the same as ethanolamine. The value of a_L for liposomes was 44.8 Å^2/lipid. Liposome concentration in solution can be calculated by dividing the total lipid concentration by N_{tot}.

Fluoroimmunoassay for Tetanus, Botulinum, and Cholera Toxins. The inner 60 wells of a 96-well Immulon 4 plate were coated with monoclonal anti-tetanus toxin C fragment, monoclonal anti-botulinum A toxin C fragment, or monoclonal anti-cholera toxin B subunit for assays for tetanus, botulinum, and cholera toxin fragments, respectively. Coating solution (150 μL) containing 10 μg/mL antibody in 50 mM carbonate/bicarbonate buffer (pH 9.6) was dispensed in each well. After being covered with a plate sealer, the plate was incubated at 4 °C on a plate shaker at 600 rpm for 18 h. The coating solution was aspirated using an automated plate washer (Biotek Instruments), and the wells were blocked with 300 μL/well of 1 wt % BSA (RIA grade) for 3 h at 4 °C. The wells were washed with PBS twice and then exposed to dilutions of the appropriate toxin fragment or buffer (control). After incubation at 37 °C for 1 h, wells were washed with PBS four times. Liposome solution was dispensed into the wells, and the microtiter plate was incubated again for 1−2 h at 37 °C. Wells were washed six times with PBS to remove the unbound and nonspecifically bound liposomes. Lysis buffer containing a detergent, Triton X-100 at 10 mM in borate buffer (pH 9), was added, and the plate was shaken on the plate shaker for 15 min at 600 rpm. The fluorescence signal was read in a fluorescence plate reader (Cambridge Instruments, Watertown, MA) using an excitation filter of 550 nm and emission filter of 580 nm.

Nonspecific Binding Experiments. To reduce the nonspecific binding of toxins and liposomes in the immunoassays, the following parameters were optimized: blocking protein, coating buffer, wash buffer, type of microtiter plate, diluents for the different steps, and incubation time. The optimum choice of these parameters is dependent on the antigen−receptor system being considered, and hence, these studies had to be repeated for each toxin. Various concentrations and grades of casein, BSA, and gelatin were considered as blocking proteins and diluents. The different polystyrene microtiter plates tested to maximize antibody adsorption and minimize well-to-well variations were Costar high binding (Corning Costar, Cambridge, MA), Immulon high binding 3 and 4 (Dynatech Laboratories, Chantily, VA), and Nunc Maxisorp (Nalge Nunc International, Rochester, NY). Different incubation times and temperatures were tested for incubating the wells with monoclonal antibodies, toxins, and liposomes to maximize signal-to-noise ratio.

RESULTS AND DISCUSSION

Properties of Fluorescent Liposomes. Fluorescent liposomes were prepared with the ganglioside receptor for tetanus toxin, botulinum toxin, or cholera toxin incorporated in the bilayer together with phospholipids (DHPE) conjugated with a marker rhodamine. Liposomes had hydrodynamic diameters of 120−130 nm and contained about 9500−11 000 ganglioside molecules and 19 000−22 000 rhodamine molecules. Table 2 lists the physical properties of liposomes. At neutral pH, the sialic acids in the headgroup of GT1b or GM1 and rhodamine moieties in rhodamine-DHPE are negatively charged. Since DSPC and cholesterol (the other constituents of bilayer) are zwitterionic and uncharged, respectively, at neutral pH; liposomes carry a net negative charge and are stabilized by electrostatic repulsion in a suspension. Liposome stability was monitored by measuring the hydrodynamic diameter and the lipid loss, and no significant change (<5%) was observed over one year. For long-term storage, liposomes were suspended in the same buffer that was used for preparing them to ensure that osmolarity of the solutions inside and outside of a liposome is the same. This avoids osmotic pressure-induced size changes such as swelling and bursting of liposomes. GT1b in the total lipids was always maintained at less than 5 mol % as its large headgroup may destabilize liposomes. Each liposome carried ~10 000 ganglioside molecules, probably well in excess of what is required for binding to a toxin molecule. But, since one liposome, owing to its size, can bind to multiple toxin molecules simultaneously, ganglioside density was kept high. Multivalent binding can lead to significantly higher binding constants,[23] allowing smaller concentrations to be detected. The concentration of rhodamine-DHPE was kept to a maximum of 15%. Theoretically, it is possible to increase signal generated by a liposome by incorporating higher concentrations of rhodamine-DHPE, but incorporation of higher concentrations of rhodamine-DHPE in the bilayer resulted in unstable liposomes. It has been reported that phosphatidylethanolamine (PE), owing to its smaller headgroup, prefers to form planar bilayers rather than curved ones and its presence in large concentrations can destabilize a spherical liposome.[24]

Table 2. Properties of Fluorescent Ganglioside-Bearing Liposomes

	GT1b liposomes	GM1 liposomes
hydrodynamic diameter	120 nm	128 nm
conentration of liposomes in suspension	1.57×10^{-8} M	1.37×10^{-8} M
ζ potential (in 10 mM KCl)	−38.7 mV	−32 mV
no. of receptors (GT1b or GM1)/liposome	9.6×10^3	1.1×10^4
no. of rhodamine molecules/liposome	1.9×10^4	2.2×10^4

Fluorescence Quenching in Liposomes. The rhodamine molecules in the liposomes are present in relatively high surface concentration and undergo self-quenching. There have been a number of mechanisms postulated for self-quenching of rhodamine at high concentrations including formation of nonfluorescent dimer, energy transfer from monomer to dimer, and collisional quenching. With rhodamine-DHPE, the collisional quenching can be ruled out as the fluors are too far apart to collide within a fluorescence lifetime of a few nanoseconds.[25] Therefore, formation of a nonfluorescent dimer and energy transfer without emission to the dimers account for the concentration quenching of rhodamine in liposomes.[25,26] The extent of quenching was determined by lysing (and hence diluting) liposomes with the nonionic

(22) Israelachvili, J. N.; Mitchell, D. J. *Biochim. Biophys. Acta* **1975**, *389*, 13−19.

(23) Crothers, D. M.; Metzer, H. *Immunochemistry* **1972**, *9*, 341−357.

(24) Israelachivli, J. N. *Intermolecular and Surface Forces*; Academic Press: New York, 1995; pp 366−394.

(25) MacDonald, R. I. *J. Biol. Chem.* **1990**, *265*, 13533−13539.

(26) Chen, R. F.; Knutson, J. R. *Anal. Biochem.* **1988**, *172*, 61−78.

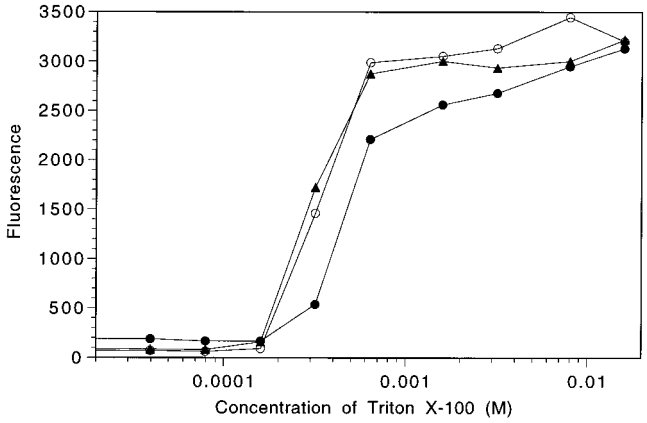

Figure 3. Fluorescence dequenching of liposomes upon lysis by a nonionic surfactant: (▲) GM1 liposomes; (●) GT1b liposomes; (○) control liposomes. Rhodamine molecules are up to 97% quenched in intact liposomes. Disruption of liposomes by a surfactant leads to dequenching of fluorescence. The steep rise in fluorescence (indicating onset of liposome lysis) begins when surfactant concentration reaches the critical micellar concentration (0.22 mM for Triton X-100).

surfactant Triton X-100. The liposome concentration was maintained constant at 20 μM total lipids, and the surfactant concentration was increased from 0 to 20 mM. The results are presented in Figure 3. The intact liposomes yielded a signal 30 times lower than the case in which liposomes were completely disrupted, indicating that rhodamine molecules were quenched by up to 97% in an intact liposome. Hence the fluorescent liposomes, when used in an immunoassay, need to be disrupted prior to signal measurement to maximize the fluorescence signal. Figure 3 shows the fluorescence dequenching of liposomes upon addition of a surfactant. The initial increase in fluorescence upon surfactant addition is gradual until the critical micellar concentration (cmc) of Triton X-100 (0.22 mM) is reached. Dequenching in this region results from the intercalation of surfactant monomers into liposomes, which decreases the average surface concentration of rhodamine-DHPE. As the concentration of surfactant is increased beyond the cmc, liposomes begin to break apart to form micelles leading to a rapid dilution of rhodamine-DHPE molecules. This region is marked by a rapid increase in the fluorescence signal. Once the surfactant concentration is large enough to solubilize a majority of the liposomes, increase in fluorescence upon surfactant addition is marginal. Finally, a saturation point is reached at a Triton X-100 concentration of ~10 mM at which the fluors are completely dequenched. On the basis of these findings, 10 mM Triton X-100 was used to disrupt liposomes prior to fluorescence measurement in the sandwich immunoassay.

Nonspecific Binding of Liposomes. Liposomes, depending on their composition and groups present on the outer surface, can exhibit high nonspecific binding to proteins and hydrophobic surfaces.[26] Although, liposomes have a very hydrophilic exterior, in the presence of highly hydrophobic surfaces the spherical bilayer can unravel, thereby exposing the hydrophobic lipid tails. This feature has been used advantageously to deposit lipid bilayers on hydrophobic surfaces by incubating them with liposomes.[28] Proteins can adsorb to liposomes by insertion of hydrophobic segments in the bilayer or by electrostatic attraction if liposomes are charged.[29,30] BSA (1 wt % in PBS, pH 7.4, RIA grade) performed the best as a blocking agent to minimize adsorption of liposomes (and toxin proteins) to hydrophobic polystyrene wells. BSA has a p*I* of ~4.9 and hence at pH 7.4 adsorbed BSA on polystyrene is negatively charged. Since the liposomes used were negatively charged (ζ potential ~−40 mV, Table 2), electrostatic repulsion is a probable cause of low nonspecific binding to a BSA-treated surface. BSA (0.1% in PBS) was also added to the buffer used to make dilutions of toxins and liposomes to reduce NSB during incubation steps. Immulon high-binding 4 microtiter plates performed the best in obtaining reproducible and high antibody loadings.

Fluoroimmunoassays for Tetanus, Botulinum, and Cholera Toxins. GT1b-bearing fluorescent liposomes were used in heterogeneous sandwich immunoassays for detection of the C fragment of tetanus and botulinum toxins. For detection of the B subunit of cholera toxin, GM1-bearing fluorescent liposomes were used. Panels a−c of Figure 4 show the results of sandwich immunoassays for detection of tetanus toxin, botulinum toxin, and cholera toxin, respectively. The concentration of liposomes used in an assay was kept sufficiently high to bind all toxin molecules even at the highest toxin concentration and was determined by binding-isotherm experiments (data not shown). In Figure 4, the fluorescence signal in the immunoassays increases with increasing toxin concentration until a saturation point is reached at high toxin concentrations. The saturated fluorescence signal is reached when either (1) the entire population of antibody sites is saturated with toxin or (2) the entire well surface is covered with a monolayer of liposomes. In the present case, the later scenario is more likely and hence, if desired, the upper bound of the working assay range can be extended by using a lower concentration of liposomes. The fluorescence versus toxin concentration plots have a sigmoidal shape, typical of a sandwich immunoassay. The data were fit by a four-parameter logistic model of the form[31]

$$F = \beta_2 + \frac{\beta_1 - \beta_2}{1 + (x/\beta_3)^{\beta_4}}$$

where F is the fluorescence signal and x is the toxin concentration. β_1 and β_2 are the asymptotic signals as $x \rightarrow 0$ and $x \rightarrow \infty$, respectively. β_3 is the predicted concentration at the response halfway between the two asymptotes, and β_4 is related to the slope. The logistic model fit the experimental data reasonably well as indicated by the R^2 values of 0.996, 0.998, and 0.993 for the assays for tetanus, botulinum and cholera toxins, respectively. For quantitation of immunoassays, the minimum detectable concentration (MDC) was defined as the lowest concentration of analyte that results in an expected fluorescence signal that is two standard deviations higher than the mean response at zero concentration. The MDC values were in the nanomolar range for all three toxins as listed in Table 3. β_3 can be used[32] as the apparent dissociation

(27) Jones, M. N. *Curr. Opin. Colloid Interface Sci.* **1996**, *1*, 91−100.
(28) Puu, G., Gustafson, I. *Biochim. Biophys. Acta-Biomembr.* **1997**, *1327*, 149−161.
(29) Bergers, J. J.; Vingerhoeds, M. H.; Vanbloois, L.; Herron, J. N.; Janssen, L. H. M.; Fischer, M. J. E.; Crommelin, D. J. A. *Biochemistry* **1993**, *32*, 4641−4649.
(30) Malmsten M, *Colloids Surf. A-Physicochem. Eng. Aspects* **1999**, 159, 77−87.
(31) O'Connell, M. A.; Belanger, B. A.; Haaland, P. D. *Chemom. Intell. Lab. Syst.* **1993**, *20*, 97−114.
(32) Azimzadeh, A.; Van Regenmortel, M. H. V. *J. Mol. Recognit.* **1990**, *3* (3), 108−116.

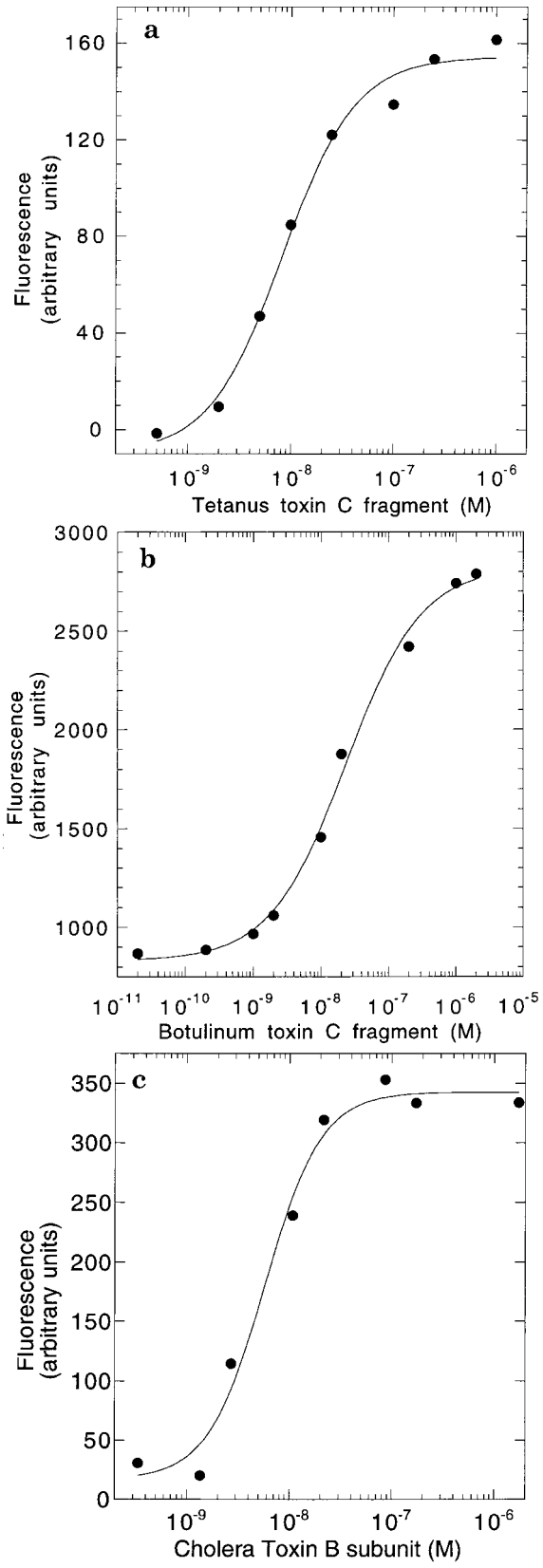

Figure 4. Sandwich fluoroimmunoassays for (a) tetanus toxin C fragment using GT1b liposomes, (b) botulinum toxin C fragment using GT1b liposomes, and (c) cholera toxin B subunit using GM1 liposomes. GT1b is a ganglioside that binds specifically to tetanus and botulinum neurotoxins. GM1 is a highly specific receptor for cholera toxin. The solid line represents the curve fit using the logistic model. The results of immunoassays are listed in Table 3.

Table 3. Results of Fluoroimmunoassays Using Ganglioside-bearing Liposomes for Detection of Bacterial Toxins[a]

	assay for TTC	assay for BTC	assay for CTB
apparent K_d (M^{-1})	8.3×10^{-9}	2.3×10^{-8}	5.7×10^{-9}
MDC (M)	1.2×10^{-9}	1.2×10^{-9}	1.5×10^{-9}

[a] TTC, tetanus toxin C fragment; BTC, botulinum toxin C fragment; CTB, cholera toxin B subunit; K_d, dissociation constant; MDC, minimum detectable concentration.

constant, and the values are listed in Table 3 as well. The apparent dissociation constants are in the 10^{-8} M range, indicating relatively strong binding of ganglioside-bearing liposomes to toxins.

CONCLUSIONS

In the ever-advancing field of biosensors and immunoassays, there is always a desire for flexibility in the types of ligands that can be used for molecular recognition. Proteins, especially antibodies, have been the receptor of choice for most applications. Recently, researchers have started to explore alternative receptors such as combinatorial peptides and aptamers. Glycolipids have long been known as receptors for a variety of biological molecules but have not been used extensively in development of biosensors and immunoassays. The major reasons are their low solubility in water and their small size, which makes it impractical to do any covalent modification without adversely affecting their binding. But glycolipids can easily be incorporated in self-assembled structures such as liposomes. Liposomes possess a number of features that make them attractive as assay reagents. They provide a large internal volume and outer surface area where molecules can be entrapped or attached; they provide a very flexible, cell membranelike environment where biological molecules can maintain their native conformation; they form a stable suspension in water; and they can be tailor-made to offer sites for covalent chemistry or decrease nonspecific binding. We prepared liposomes containing gangliosides (receptors for bacterial toxins) to develop sensitive immunoassays for these toxins. Signal generation was achieved by incorporating thousands of fluorophore−lipid conjugates in liposomes. The fluorescent liposomes were employed for detection of tetanus, botulinum, and cholera toxins and as low as 1 nM of each toxin could be detected. Furthermore, as these liposomes do not contain any protein component, they have vastly superior shelf life than antibody−marker conjugates typically used in immunoassays and biosensors.

The present approach offers a generic platform to employ membrane receptors in biosensing and immunoassays. Many bacterial toxins and viruses use lipids and membrane proteins on a cell surface as their specific receptors. Consequently, liposomes containing suitable lipids or membrane proteins can be used as sensitive probes for a variety of analytes of significance in clinical diagnostics, food quality monitoring, and biological warfare reagent detection.

Received for review July 27, 2000. Accepted October 6, 2000.

AC000846L