Optical Signal Transduction Triggered by Protein−Ligand Binding: Detection of Toxins Using Multivalent Binding

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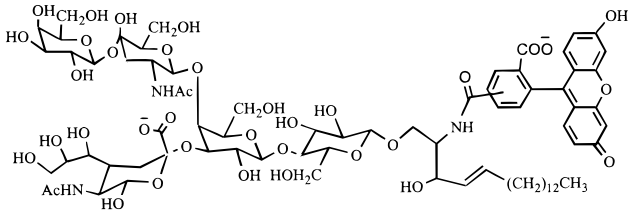
Specific multivalent binding between bacteria toxins and oligosaccharide receptors on a host cell surface is a paradigm for protein−sugar interactions. One of the best-characterized recognition pairs is cholera toxin (CT) and ganglioside GM1.[1] CT consists of one A subunit responsible for catalysis and five B subunits, which define the binding region. Toxicity is initiated by the recognition and binding of B subunits to the pentasaccharide moiety of GM1 in the cell surface followed by a mechanism involved in the entry of A subunit through the membrane into the cell.[2] A variety of techniques have been explored to examine the interaction between CT and GM1, including ^{125}I labeling of CT binding to cells,[1a,b,3] radiolabeled immunoassay,[4] and flow cytometry[5] using fluorophore-labeled CT. Recently, surface plasmon resonance (SPR)[6] has been applied to investigate the binding affinity and specificity of CT with GM1, either using self-assembly hybrid bilayers or liposomes immobilized on the surface of SPR devices. A direct colorimetric detection of a receptor−ligand interaction by a polymerized bilayer assembly was also reported.[7] Although the toxin labeling and SPR provide useful tools for studying the binding interaction between toxins and receptors, they have serious limitation in terms of toxin sensing. Toxin labeling is not practical for real sensing of toxins while SPR and colorimetry suffer low sensitivity, nonspecific binding (SPR), and slow response (colorimetry). A recent advance in biosensing was achieved by direct coupling of a biological recognition event with signal transduction and amplification−ion-channel switches, carefully designed to anchor in a rather complicated self-organized structure.[8] Here we report a general fluorescence transduction method sensitive to multivalent binding as a scheme for toxin detection. The transduction method can achieve an agent-free assay which is fundamentally different from competitive-binding assay method based on fluorescence quenching of ligands held in close proximity by multivalent receptor reported recently.[17] This system couples the specific multivalent reaction between toxins and receptors with distance-dependent fluorescence self-quenching of fluorescein. Such a transduction scheme possesses many advantages over the approaches reported so far including relatively high chemical and functional stability of the receptor and transduction element, high specificity and enhanced sensitivity, and possible on-line measurement[9] and remote sensing using optical fiber techniques.[10] The key advantage of directly coupling recognition and signal transduction is the amplification of specific versus nonspecific binding events.

Scheme 1

F-GM1



The transduction element used in this design is fluorescein, which has high extinction coefficient, high fluorescence quantum yield, and proximity-dependent fluorescence self-quenching. Fluorescein is covalently attached to the free amino group of lyso-GM1 by coupling lyso-GM1 with 5-(and 6-)-carboxyfluorescein, succinimidyl ester, in a mixing solvent of DMF and Na_2CO_3 buffer (pH = 7.4) to produce a fluorescein-labeled GM1 (F-GM1, Scheme 1).[12] The fluorescein should have minimal influence on the binding affinity of the pentasaccharide moiety of GM1 to CT as the binding strength originates mainly from the hydrogen bonding located on sugar moieties and the fact that the two alkyl chains act as an anchor on the membrane surface.[11] Strong fluorescence (excited at 498 nm and monitored at 520 nm) without self-quenching is observed for F-GM1 dissolved in tris-buffer (pH = 8.0, [F-GM1] ≤ 50 nM) or distributed in the outer surface of the vesicles of palmitoyl, 9-octadecenoylphosphatidycholine (POPC) ([POPC]/[F-GM1] ≥ 200).[8,13] This indicates that F-GM1 is homogeneously distributed with no aggregation.

The strong fluorescence decreases dramatically upon addition of CT and the degree of fluorescence quenching depends on the concentrations of both CT and F-GM1 as shown in Figure 1 for F-GM1 in the outer surface of POPC vesicles. In tris-buffer, similar results are observed.[18] The detection limit can easily reach an order of 100 pM using conventional fluorimeter (a SPEX Flurolog-2 Spectrofluorimeter).[19] In contrast, a much smaller

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(14) There are two possible mechanisms for the small fluorescence decrease of the F-GM1 with addition of albumin: (1) the interaction of albumin with the surfaces of POPC vesicles causes reorganization of the bilayer structure, inducing redistribution and partial aggregation of F-GM1; (2) weak binding of F-GM1 with albumin slightly quenches the fluorescence of F-GM1. The second mechanism is more likely as demonstrated by the observation that the fluorescence intensity of F-GM1 in tris-buffer decreases more than in POPC vesicles upon addition of albumin.

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(18) In the surface of POPC vesicles, the lateral diffusion plays an important role.[8] In tris-buffer, the homogeneously dissolved F-GM1 (<30 nM) can also diffuse to accommodate multivalent binding to CT.

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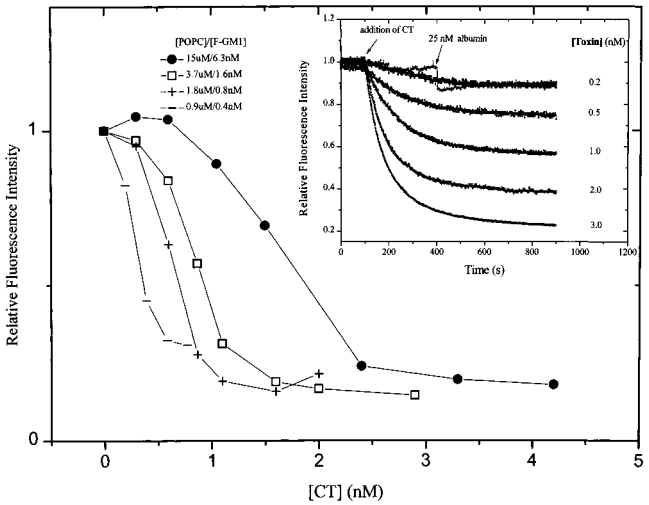

Figure 1. Relative fluorescence of F-GM1 in POPC vesicles as a function of [CT]. Measurements were taken 15 min after addition of CT. (inset) Fluorescence intensity change of F-GM1 (2 nM) in POPC vesicles (1 μM) upon addition of different concentration of CT and albumin.

fluorescence decrease is observed for the interaction of the F-GM1 in POPC vesicles and tris-buffer with a relatively high concentration of albumin.[14] Comparison with flow cytometry results[5] obtained with a labeled CT and an unlabeled GM1 in POPC bilayers coated on the surface of glass beads shows little difference in binding affinity, demonstrating that the high specificity of the binding between the pentasaccharide moiety and CT is not significantly affected by the labeled fluorescein. We also have investigated the binding reaction of F-GM1 with B subunit of CT and similar results are obtained.

The fluorescence decrease is attributed to the self-quenching due to the close proximity of fluorescein fluorophores of F-GM1 brought by the multivalent toxin. Addition of an excess of CT into F-GM1 in tris-buffer or POPC vesicles results in smaller decrease of fluorescence due to the formation of more monovalent complexes at the expense of multivalent complexes. For instance, addition of 2 nM CT in 2 nM F-GM in POPC vesicles (10 uM) results in 80% decrease in fluorescence intensity while 100 nM CT causes only 40% decrease. On the basis of crystal structure,[15] the pentameric saccharide has a diameter of ~60 Å and a central pore of ~20 Å wide. The binding of F-GM1 to CT is expected to bring fluoresceins within a critical self-quenching distance of ~50 Å for fluorescein. This system also provides a convenient method to investigate the multivalent interaction between receptors and toxins, such as real time kinetics and binding affinity in both homogeneous solution and in the biomimetic surface of lipid vesicles. We are currently trying to establish a model for quantitative analysis of the binding kinetics and affinity between F-GM and CT, features which are difficult to measure without directly labeling the toxin molecules.

Besides the application of this system to study the interaction between toxins and receptors, it also can be utilized to construct near real time optical biosensors for toxins with high sensitivity and specificity. The labeled F-GM1 bound to toxins can be regenerated by inducing toxin dissociation via addition of unlabeled GM1 as shown in Figure 2. The regeneration mechanism and kinetics of F-GM1 are complicated and probably determined by many factors such as the aggregation of GM1, incorporation of GM1 into lipid bilayers, and lateral diffusion. Toxin dissociation from F-GM1 is much slower than the association due to the multivalent nature of binding. This is consistent

(19) The sensitivity strongly depends on the concentration of F-GM1. Lower [F-GM1] can achieve a higher sensitivity (see Figure 1). The concentration used in the inset of Figure 1 is not the lowest concentration for a reliable detection; therefore, the detection limit of 0.2 nM shown in the figure is not the lowest detection limit.

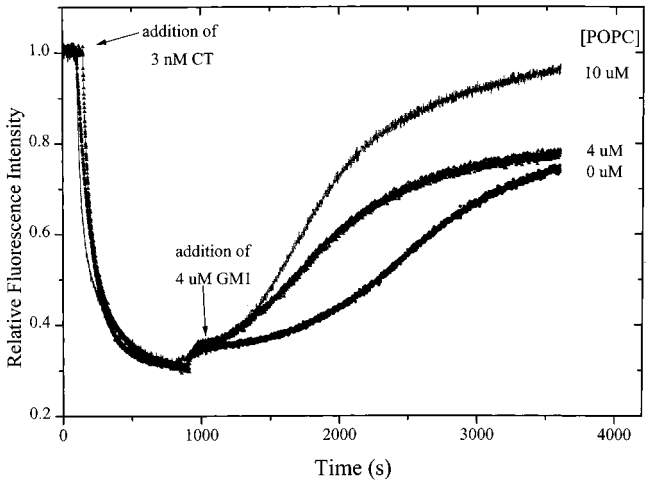

Figure 2. Fluorescence change of F-GM1 (2 nM) in aqueous solution and the surface of POPC vesicles upon addition of CT and GM1.

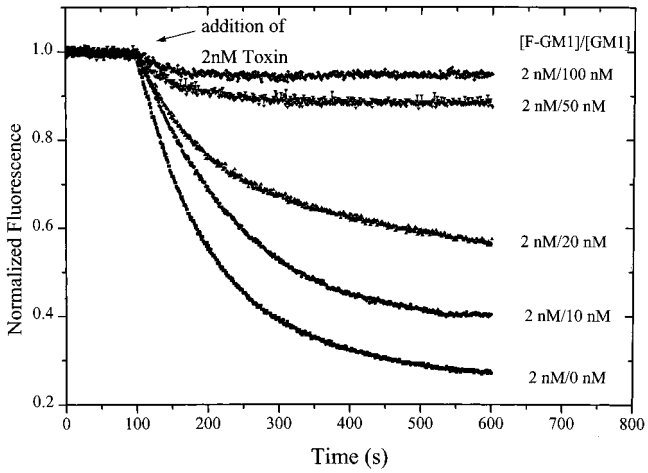

Figure 3. Competitive binding of CT with F-GM1 and GM1 in POPC vesicles (10 μM).

with the results obtained by other techniques.[6,16] The kinetics of the displacement of F-GM1 by GM1 depends on the concentration of POPC vesicles. Competitive binding of CT with F-GM1 and GM1 in the biomimetic surface of POPC vesicles is shown in Figure 3. As expected, the presence of GM1 inhibits the binding of CT to F-GM1 to result in a smaller decrease of fluorescence. Similar competitive binding is observed for CT with F-GM1 and GM1 in tris-buffer solution.

As described above, the fluorescence self-quenching mechanism as a transduction method can be applied to take advantage of multivalent binding for investigation of receptor−protein interaction and other types of multivalent interaction. The technique is very flexible and sensitive and can be used in a wide range of applications for detection of toxins and other molecules in both homogeneous solutions and the biomimetic surfaces of vesicles. Such an approach is expected to work using monolayers, bilayers, and multilayers immobilized on substrates such as microspheres and other solid surfaces to act as a sensitive optical sensor. Moreover, multivalent receptor ligands can be replaced by monovalent coreceptor in application to other recognition ligands (e.g., RNA, DNA, or polypeptides). We are currently investigating incorporation of the labeled receptor into immobilized monolayers and bilayers on solid substrates to construct an optical sensing device for toxin detection.

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