Optical Biosensor Based on Fluorescence Resonance Energy Transfer: Ultrasensitive and Specific Detection of Protein Toxins

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Development of new methods for detecting biological toxins and pathogens is critical to the diagnosis of pathogenic diseases and for environmental sensing to counter the use of biological agents. Two major approaches, namely immuno-based assay [1−5] and DNA sequencing schemes,[6−8] have received extensive attention. The immuno-based assays are difficult to implement in field applications owing to poor stabilities of antibodies and the need for unstable reagents. DNA sequencing techniques are inherently slow and instrument-intensive and cannot meet many requirements for practical field use. Here we apply another relatively undeveloped concept, structure−function dependent properties of the cell surface receptors targeted by biological toxins or pathogens,[9−11] to develop a general, reagent-free, highly sensitive and specific sensing technology for effective detection of toxins through distance-dependent fluorescence energy transfer induced by multivalent interactions.[12,13]

Current biosensor approaches often do not build in one of the most important aspects of biological signaling processes—the direct coupling of a recognition event with signal transduction and amplification.[14] Recently, a gated ion channel biosensor was reported that can be switched on by a protein binding event.[5] The approach described here represents an analogous optical biosensor where mobility of optically tagged receptors in the upper leaf of a bilayer membrane is used to trigger a two-color optical fluorescence change upon protein binding.

We recently reported[15] an optical method for detection of cholera toxin (CT) through fluorescence self-quenching. Although we achieved high sensitivity, the GM1 tagged with fluorescein cannot be stably anchored in the biomimetic surface of phospholipid bilayers due to the ionic nature and high water solubility of the fluorescein. Another drawback is its pH-dependent fluorescence. The possible direct exposure of the fluorescein to interfering species is most likely responsible for the nonspecific binding signal observed for albumin. This appoach, where recognition is signaled by a decrease in fluorescence intensity, cannot be easily distinguished from other interfering phenomena (e.g., temperature-induced decrease in fluorescence or fluorescent samples).

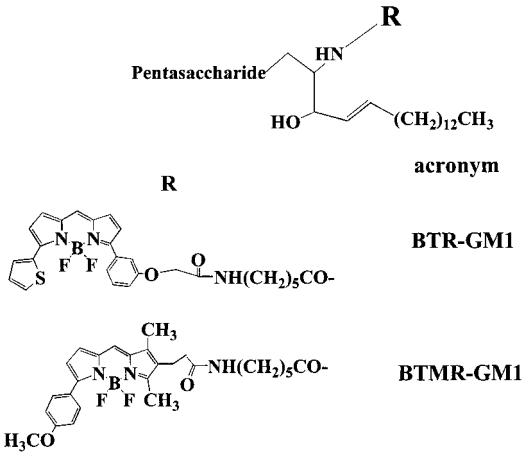

Figure 1.

To overcome the problems associated with the previously reported method, we directly couple the binding event with fluorescence resonance energy transfer (RET) to achieve a simultaneous two-color change. By using nonpolar, pH-insensitive fluorophores as probes, the labeled receptors can be stably incorporated into the hydrophobic interior of the bilayers, and the detection selectivity can dramatically be improved by preventing any possible direct contact between the fluorophores and intereferring species. In this system, the signal transduction responds only to the events which induce receptor aggregation. The combination of a specific interaction with a selective transducer dramatically enhances the discrimination against nonspecific bindings. The fluorophore-labeled receptors are shown in Figure 1 (see ref 15 for the structure of the pentasaccharide) and can be asymmetrically incorporated into a biomimetic membrane surface[16] either in the outer layer of the preformed vesicles (10−50 nm in diameter)[20] of phospholipids such as palmitoyl, 9-octadecenoyl phosphatidylcholine (POPC) or in the lipid bilayer on supporting glass beads.[21] These bilayer membrane surfaces retain the dynamic and structural properties of cell membranes so that the labeled receptors can maintain their mobility on the membrane surfaces.

BTMR-GM (donor) and BTR-GM1 (acceptor) were chosen as the energy transfer pair for the following properties of BODIPY fluorophores:[17] (a) neutral and hydrophobic; (b) pH insensitive for both absorption and fluorescence spectra; (c)[17] strong absorption and high fluorescence quantum yield; (d) significant overlap

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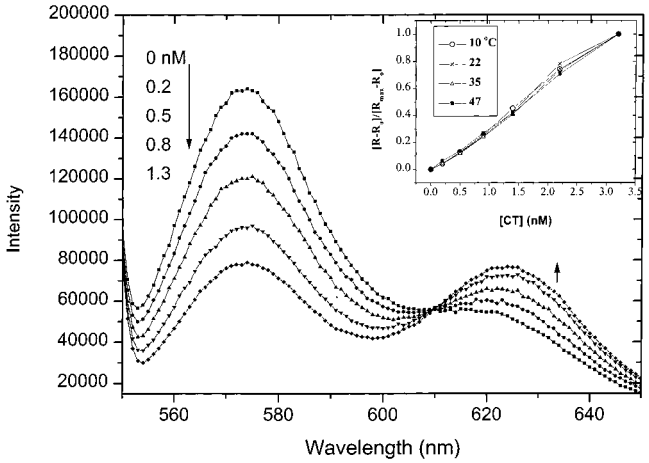

Figure 2. Fluorescence spectra (ex. at 530 nm) of BTR-GM1 and RTMR-GM1 in the outerlayer of POPC bilayers on glass beads with different concentration of CT. Sample preparation: 5 mg of glass beads coated with POPC bilayers were incubated in 31 μL of BTR-GM1 (324 nM) and 28 μL of BTMR-GM1 (356 nM) aqueous solution for overnight. After they were washed three times, the beads were suspended in 1 mL of Tris-buffer. The sample contains 120 μL of beads diluted to 240 μL. (inset) The plot of $(R - R_0)/(R_{max} - R_0)$ versus [CT] at different temperatures. R, R_0, and R_{max} are the intensity ratio (I_{624}/I_{574}) of the two fluorescence peaks at 624 nm (acceptor) and 574 nm (donor) for the samples with CT, sample without CT, and sample with saturating CT, respectively.

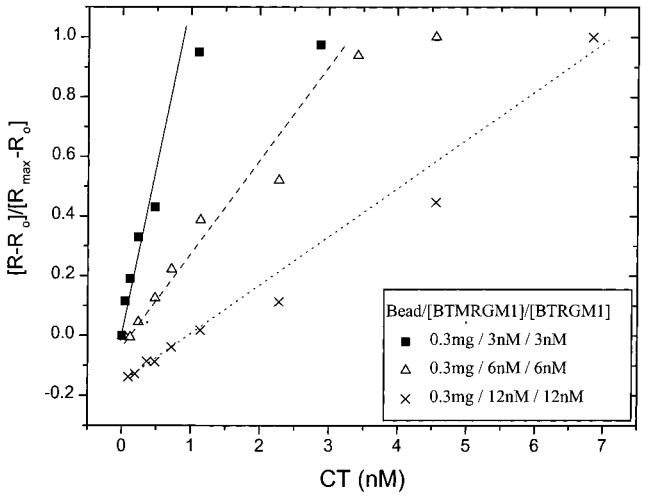

Figure 3. Receptor aggregation induced RET measured by flow cytometry. Glass microspheres coated with a bilayer of POPC and the labeled receptors were incubated with various concentrations of CT at room temperature for 30 min before flow cytometry measurement. Microsphere fluorescence was excited at 514 nm with an argon ion laser and donor and acceptor fluorescence was collected through band-pass filters and detected with photomultiplier tubes. Analogue detector signals were processed with a variable gain ratio module to give the ratio of acceptor to donor fluorescence on a particle by particle basis. Data were normalized by subtracting the ratio of samples before addition of CT (R_0), and expressing the result as a fraction of the maximal ratio at saturating CT (R_{max}).

of the BTMR-GM1 fluorescence spectrum with the absorption spectrum of the BTR-GM1. The nonpolarity of the fluorophores is important for stable anchorage in the hydrophobic interior of the phospholipid bilayers and high signal transduction selectivity by avoiding possible direct interaction of the probes with interfering species. Figure 2 shows the fluorescence spectral change upon addition of different [CT]. The fluorescence intensity (peak at 624 nm) of BTR-GM1 increases at the expense of the fluorescence intensity (peak at 574 nm) of BTMR-GM1. Without CT, the donor- and acceptor-labeled GM1 are distributed homogeneously in the membrane surface and fluoresce independently when the surface density of the receptors is low ([POPC]/[receptors] > 200).

The aggregation of the labeled receptors induced by the multivalent binding of CT brings the energy transfer pair into close contact for an efficient RET to occur. As shown in Figure 2 (inset), the normalized ratio (NR, defined as $(R - R_0)/(R_{max} - R_0)$) related to the fluorescence intensity change for both donor and acceptor has a linear relationship with the concentration of CT within the upper detection limit (approximately $^1/_5$ of the total labeled receptor concentration, which is consistent with the five binding sites of each CT and almost all of the receptors bind to the CT). The detection sensitivity and dynamic range can be adjusted by the total concentration of the labeled receptors. Lower concentration of the labeled receptors gives higher sensitivity but smaller detection range. Less than 0.05 nM of CT can be reliably detected with a response time of less than five minutes. The NR starts to level off beyond the upper detection limit due to saturation of the receptor and then drops slowly with further addition of CT. The parameter drop is reasonably attributed to the formation of low-valent complexes due to the presence of excess concentration of CT. As expected by the fact that the hydrophobic fluorophores should anchor in the interior of the membrane, high concentration of albumin (more than 10^3 times higher than the toxin detection limit) causes no change in the fluorescence spectra.

Such a detection scheme using multiple fluorescent probes with similar photophysical properties has a huge advantage over single signal systems and shows little temperature dependence over the tested range of 10 °C to 47 °C (inset of Figure 2). The insensitivity to temperature can be understood by the fact that the NR is taken from two similar fluorophores and they act as an internal reference to offset any absolute intensity change caused by temperature variation or possibly other environmental changes. Another advantage of the distance-dependent signal transduction over techniques based on changes in the index of refraction, such as surface plasmon resonance spectroscopy, is its silence to the nonspecific binding of toxin itself to the membrane surfaces.

Fluorescence measurements on surfaces are compatible with a variety of detection platforms including microscopy, microplates, and flow cytometry.[18] Flow cytometry can also measure several fluorescence and light scatter signals simultaneously, making it especially useful for RET measurements. Presented in Figure 3 is the CT-induced receptor aggregation as measured by RET using flow cytometry. RET is linear with toxin concentration, and the dynamic range and sensitivity depend on the receptor density on the bilayer surface. We expect that the sensitivity of this assay can be further improved by decreasing the microsphere concentration while holding receptor surface density constant, thus lowering the concentration of receptor without sacrificing signal.

In conclusion, the receptor−toxin recognition pair coupled with the energy-transfer optical-transduction techniques described here provides a general method for the effective detection of multivalent bindings such as lectin/saccharide interactions[20] and, in particular, protein toxins. The essential elements for this approach include the construction of a biomimetic membrane surface that contains the optically labeled recognition molecules, species-specific multivalent binding, and selective signal transduction that is triggered by the binding event. Such a direct, reagent-free assay with high sensitivity and specificity and stability of the receptors and the membrane should find a wide application in laboratory and field diagnostics and sensing of selected biological toxins and pathogens.

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