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Resonant Energy Transfer Based Biosensor for Detection of Multivalent Proteins

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Abstract

We have developed a new fluorescence-based biosensor for sensitive detection of species involved in a multivalent interaction. The biosensor system utilizes specific interactions between proteins and cell surface receptors, which trigger a receptor aggregation process. Distance-dependent fluorescence self-quenching and resonant energy transfer mechanisms were coupled with a multivalent interaction to probe the receptor aggregation process, providing a sensitive and specific signal transduction method for such a binding event. The fluorescence change induced by the aggregation process can be monitored by different instrument platforms, e.g. fluorimetry and flow cytometry. In this article, a sensitive detection of pentavalent cholera toxin which recognizes ganglioside GM1 has been demonstrated through the resonant energy transfer scheme, which can achieve a double color change simultaneously. A detection sensitivity as high as 10 pM has been achieved within a few minutes (c.a. 5 minutes). The simultaneous double color change (an increase of acceptor fluorescence and a decrease of donor fluorescence intensity) of two similar fluorescent probes provides particularly high detection reliability owing to the fact that they act as each other's internal reference. Any external perturbation such as environmental temperature change causes no significant change in signal generation. Besides the application for biological sensing, the method also provides a useful tool for investigation of kinetics and thermodynamics of a multivalent interaction.

Keywords: Biosensor, Fluorescence resonant energy transfer, Multivalent interaction, Cholera Toxin, Ganglioside GM1, Signal Transduction

Introduction

Rapid, simple and cost-effective medical devices for screening medical diseases and infectious pathogens are essential for early diagnostics and improved treatment of many illnesses. In most of cases, medical diagnostics involve rapid, selective and sensitive detection of signatured biological substances (e.g. proteins, metabolites and nucleic acids, as well as microorganisms such as bacteria and viruses) in a rather complicated biological fluids or matrices. A biosensor which meets the requirements of high sensitive and specificity can provides a convenient tool for identification and detection of specific targets in a complex sample. These types of biosensors [1-10] often exploit exquisite recognition and binding systems (e.g. antibody/antigen, enzyme/substrate and DNA/DNA hybridization) to take advantage of the high binding specificity and affinity of the recognition systems created by mother nature. By coupling a sensitive signal transducer with these recognition systems, biosensors can be developed. In the last few decades, the combination of these two important elements (recognition and signal transduction) has generated a rich pool of new biosensor systems[1-10], which utilize different biological recognition and transduction methods.

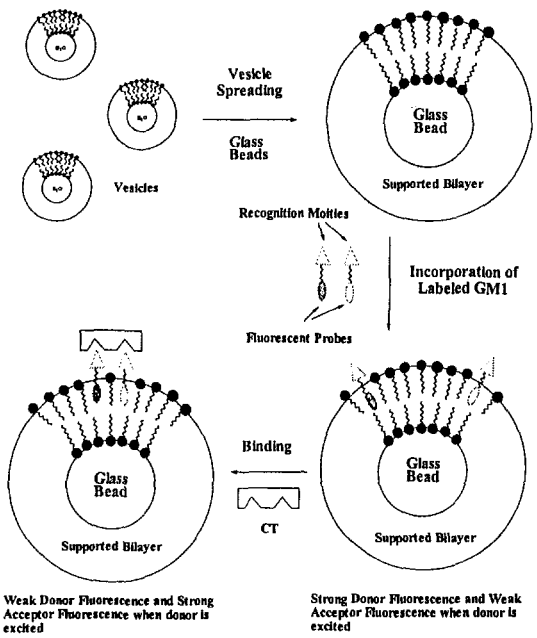
Among many signal transduction techniques, fluorescence occupies a unique position because of its flexibility for device configuration, capability for miniaturization and simultaneous imaging of a large number of samples. Another important advantage of using fluorescence for signal generation is its multiple detectable parameters which may be simultaneously altered to specific interactions. Possibility of simultaneously measuring these parameters should provide a powerful and reliable signal generation methods. In the biosensor system discussed below, we applied distance-dependent interaction between two or more fluorophores, which can be used to probe the relative distance change between these fluorescence molecules, induced by a specific aggregation process on a biomimic surface. The fluorescence properties (such as fluorescence intensity, polarization and lifetime) of the probes covalently attached to recognition

molecules respond to the distance changes of probe molecules. Such signal transduction strategies have proven to be highly successful for detection of species involved in a multivalent interaction.

We have developed three different signal transduction schemes[11-15] for detection of species involved in a multivalent interaction through a distance-dependent fluorescence self-quenching, or resonant energy transfer or combination of both. In this paper, we focus our discussions on the results obtained from resonant energy transfer. We use a specific multivalent interaction between CT/GM1 (each CT can bind up to five GM1 molecules)[16] to demonstrate the validity of our sensing design strategy.

Biosensor design

The biosensor design combines two important concepts that integrate multivalent biological recognition and fluorescence-based sensing. The multivalent biological recognition chosen for initial studies is pentavalent CT which specifically recognizes and binds five GM1 molecules. The GM1 molecules are modified through covalent attachment of a fluorescence energy transfer pair in their hydrophobic alkyl chains. Such covalent probe-labeling should not perturb its recognition capability of the penta-saccharide moities for CT, since the hydrophobic part are mainly involved in helping the GM1 molecules to anchor on the cell membrane surfaces. The labeled GM1 molecules were then incorporated into a biomimic membrane surface of natural lipids, in which the labeled GM1 molecules can undergo lateral diffusion if the membrane is fluid. If the labeled GM1 molecules are homogeneously distributed on the membrane surfaces, and the surface density is controlled to be low so that the fluorophores can fluoresce independently. In this case, a strong donor fluorescence and weak acceptor fluorescence should be observed if mainly the donor is excited. The binding of a multivalent protein such as CT with up to five labeled GM1 molecules will bring the labeled GM1 molecules, consequently both donor and acceptor, into a close proximity to trigger a RFET. The RFET will result in an increase of the acceptor fluorescence and a decrease of the donor fluorescence, providing a unique double color change simultaneously. Such a double color change is a unique indicator of the multivalent interaction such as CT/GM1. Scheme 1 describes schematically steps of the biosensor construction.



Scheme 1

Experiments

Receptor labeling

A proper amount of lyso-GM1 dissolved in 0.1 M sodium bicarbonate buffer was mixed with five equivalent of BODIPY-SE dissolved in a dry DMF. The mixture was vortexed for a few hours and TLC (CHCl3/methonol/H2O = 65/25/4 as developing solvent) showed a completion of the reaction as indicated by the disappearance of the lyso-GM1 and appearance of a new spot. The new spot was separated by TLC plate and dissolved in methanol. UV-Vis absorption spectrum of the solution was taken and the extinct coefficient constant reported in ref.17 was used to calculate the concentration of the labeled GM1. After determination of the concentration, the methanol was evaporated and re-dissolved in a certain amount of tris-buffer as stock solution. The stock solution can be stored under -20 °C for more than two years without losing activities.

Formation of supported lipid bilayers on glass microspheres with labeled GM1

Automatic vesicle spreading method was used to form supported lipid bilayers on the surface of a glass microspheres. A vesicle solution of 0.4 mM or higher in tris-buffer (pH = 8.0) formed from a probe-sonication of a thin film of phospholipid (e.g. POPC: palmitoyl, 9-octadecenoyl phosphatidylcholine) on the wall of a vial was incubated with a proper amount of glass beads overnight at room temperature (RT) under dark. The beads were washed using tris buffer four times through centrifugation. The beads were resuspened in a tris-buffer and can be stored at RT. A proper amount of beads were transferred to a vial and a proper amount of the labeled GM1 in tris-buffer was added. The mixture was then incubated at RT for a few hours and the beads were washed twice with tris-buffer. The beads were ready for use. For our experiments, the samples are usually freshly prepared within one week, although samples stored at RT for almost one month still show similar results.

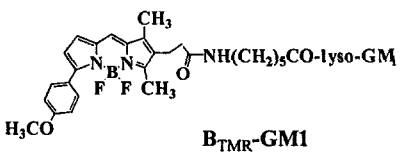
Results and Discussions

We have identified a few fluorescence energy transfer pairs which can undergo efficient energy transfer upon bringing them into a close proximity. All the energy transfer pairs work well to provide a wide range of excitation wavelengths. They are hydrophobic BODIPY dyes with large extinct coefficient and high fluorescence quantum yield. The GM1 molecules labeled with BODIPY dyes (BODIPY-GM1) are quite soluble in aqueous solution. In contrast to the highly fluorescent monomeric form of BODIPY dyes in organic solvent, the BODIPY-GM1 in aqueous solution are basically non-fluorescent, which are attributed to the formation of micelles. The fluorescence of BODIPY is self-quenched in the micellar aggregated state. When a vesicle solution of lipids or glass beads coated with supported lipid bilayers was added into the micellar solution of BODIPY-GM1, the fluorescence started to increase gradually, and then leveled off a couple of hour later at RT. The fluorescence intensity usually increases more than 15 folds. This dramatic fluorescence increase was attributed to the collapse of the BODIPY-GM1 micelles and individual BODIPY-GM1 was dissolved into the outer leaflet of the lipid bilayers [18] to reverse the fluorescence self-quenching. In the case of using lipid bilayers on glass beads, the supernatant was found to contain almost no BODIPY-GM1 after a few hours. This result clearly indicates that almost all the BODIPY-GM1 in the form of micelles are incorporated into the bilayers coated on glass beads as long as amount of the lipids used are 10 times or more. This is the basis for the estimation of the surface density BODIPY-GM1 of the bilayer surface. The above process was used to incorporate the BODIPY-GM1 into the outer layer of the supported lipid bilayer coated on a glass microspheres. We found that the BODIPY-GM1 on the outer layer of the bilayers coated on glass beads were quite stable as suggested by the findings that a few washings did not cause significant decrease in fluorescence intensity of the beads.

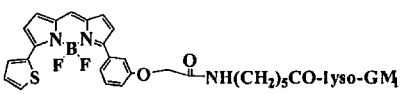
Aggregation induced Energy transfer measured by fluorimetry

We will focus our discussions on the results obtained from one energy transfer pair, B_{TMR}-GM1/B_{TR}-GM1 (see structures in scheme 2), which are imbedded into the lipid bilayers of POPC on glass beads. The results observed from other surfaces such as bilayer vesicles or bilayers on waveguide are similar. As shown in figure 1, the fluorescence of B_{TMR}-GM1 (fluorescence donor) overlaps significantly

with the excitation spectrum of B_{TR}-GM1(fluorescence acceptor). This large overlap is very important for efficient resonant energy transfer. Figure 2 shows the fluorescence spectral change of such a system upon addition of different concentration of CT. In most cases, each spectrum was recorded 5 to 10 minutes after addition of the CT. Apparently, the system gives strong donor fluorescence and weak acceptor fluorescence without CT. The acceptor fluorescence increases at the expense of the donor fluorescence upon addition of CT, induced by the aggregation process triggered by a multivalent interaction between CT/GM1. A clear isosbestic point was observed. This suggests that only two fluorescence spectra, namely fluorescence of donor and acceptor, are involved in the spectral change. The simultaneous fluorescence spectral change of donor and acceptor gives a clear indication of the presence of CT in the sample.



B_{TMR}-GM1



B_{TR}-GM1

Scheme 2

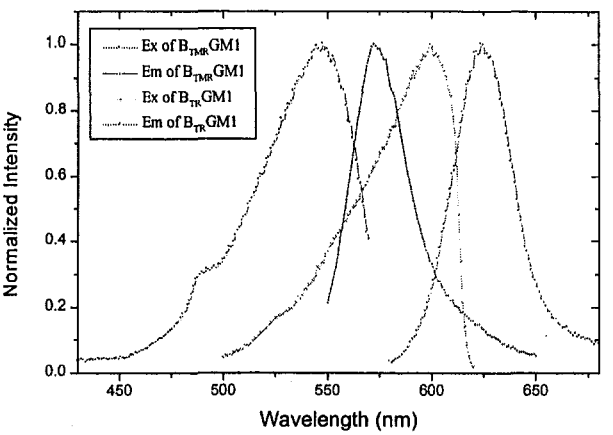


Figure 1: Fluorescence and excitation spectra of B_{TMR}-GM1 and B_{TR}-GM1 in methanol

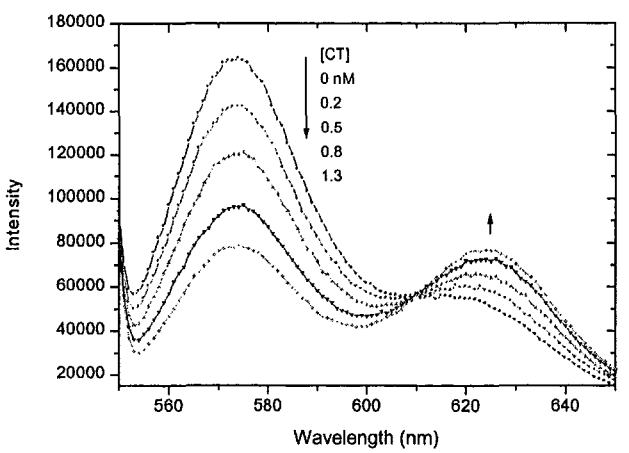


Figure 2: Fluorescence spectra (ex. at 530 nm) of B_{TR}-GM1 and $R_{TM}R$-GM1 in the outer layer of POPC bilayers on glass beads with different concentration of CT. Sample preparation: 5 mg glass beads coated with POPC bilayers were incubated in 31 μl of B_{TR}-GM1 (324 nM) and 28 μl of B_{TMR}-GM1 (356 nM) aqueous solution for overnight. After they were washed three time, the beads were suspended in 1 ml tris-buffer. The sample contains 120 μl beads diluted to 240 μl.

As expected, the detection sensitivity and dynamic range strongly depend on the total amount of the labeled GM1 in each sample since the fluorescence signal change was used compared with original fluorescence signal (background). As shown in figure 3, the extent of the signal change is proportional to the percentage of the labeled GM1 (not the absolute amount of the labeled GM1) in multivalent complexes relative to the total amount of the labeled GM1 in each sample. The samples with small amount of the labeled GM1 provide a high detection sensitivity, but small detection dynamic range, while samples with high amount of the labeled GM1 give large detection limit, but lower detection sensitivity. In the case using small amount of the labeled GM1 in a sample, small amount of target proteins can saturate all the lableled GM1 molecules available in the sample and further addition of the targets does not generate more multivalent complexes. Actually, much excess of CT (more than 50 times equivalent of upper detection limit) started to cause the fluorescence change to decrease due to the formation of low valent complexes. The upper detection limit for CT was found to be approximately one-fifth equivalent of the total labeled GM1 in the sample when the concentration of the labeled GM1 is high enough (>200 pM). This result is consistent with the pentavalency of CT for GM1. The upper detection limit was found to be higher than one fifth equivalent of the total concentration of the labeled GM1 when the concentration of the Labeled GM1 (<200 pM) is too low, suggesting that lower valent complexes are formed. In the case of this low concentration, the total fluorescence change is not as significant as the case using high concentration of the labeled GM1 upon signal saturation with enough CT. It was found that the detection sensitivity is mainly limited by the apparent binding affinity between CT and GM1, since the signal generation can be generated only when multivalent complexes are formed. Although the intrinsic binding affinity for CT/GM1 in homogeneous solution was reported to be micro-molar level, the apparent binding affinity on membrane surfaces is at least four magnitudes higher. The dramatic increase in apparent affinity compared to the intrinsic affinity in homogenous media is attributed to the co-operative behavior of multivalent binding systems in a well-defined surface, which dramatically reduces the potential huge entropy loss for the multivalent binding.

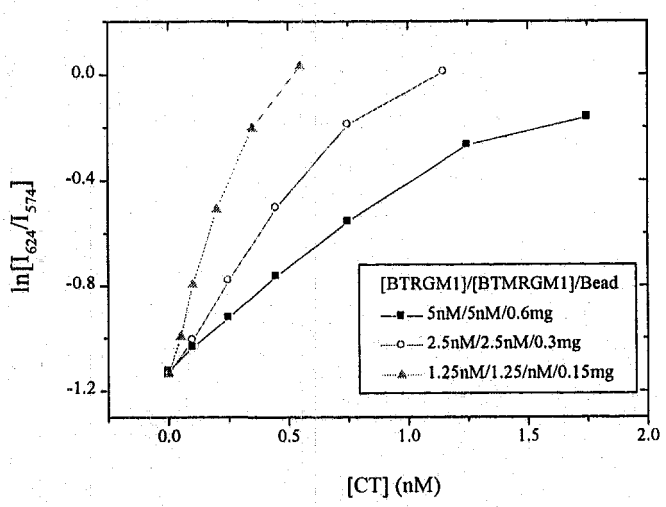


Figure 3: The plots of the fluorescence intensity ratio of acceptor/donor as a function of CT concentration for samples containing different amount of glass beads and labeled GM1

Optimization of surface density and donor/acceptor ratio

The surface density of the labeled Gm1 must be carefully controlled. In order to achieve the largest possible fluorescence change and highest detection sensitivity, the pre-binding energy transfer should be minimized and the apparent binding affinity should be maximized. Low surface density of the labeled GM1 is expected to reduce the chance for the donor and acceptor to lie close to each other for a possible energy transfer. However, the low surface density will certainly reduce the apparent binding affinity, as well as slow binding kinetics. In order to compromise the two conflicting requirements for optimal sensing performance, we tested a series of samples with different surface densities of the

labeled GM1 (both B_{TMR}-GM1 and B_{TR}-GM1) ranging from 0.02 to 4% relative to POPC. We found that the samples with the surface density from 0.1 to 0.5 % worked well and gave a large signal change for both donor and acceptor fluorescence. The samples with high density of the labeled GM1 give relatively small change probably due to the energy transfer before the binding, while relatively small signal change for the samples with low density of the labeled GM1 is attributed to the formation of low-valent complexes due to the limitation of apparent binding affinity.

Another important parameter which can dramatically influences the sensor's performance is the ratio of the donor/acceptor-GM1 on the membrane surface. We found that, although the system works well for samples with the ratio of [B_{TMR}-GM1]/[B_{TR}-GM1] from 1/3 to 3/1, the sample with [donor-GM1]/[acceptor-GM1] =1/1 gives best results. As expected, for the samples with too much donor, the percentage change of donor fluorescence intensity decreases with relatively large percentage change of acceptor fluorescence (but low absolute fluorescence intensity change), while too much acceptor relative to the donor causes small percentage change of acceptor but relatively large percentage of donor fluorescence. In both cases, the change of the ratio of the donor/acceptor fluorescence (Δ I_{624}/I_{574}) is relatively small.

Detection specificity and speed

High specificity is required for selective detection of a particular species in a complex sample to provide reliable results. The biosensor system discussed above indeed provides high specificity for the detection of CT as has been demonstrated by the observation that presence of a large quantity of albumin and peanut lectin causes little interference. We also used the biosensor system to test some samples made by mixing the water from local pond with commercial CT. The detection results are almost identical to the sample with pure CT. Besides some possible interfering species from the samples, another important potential interfering factor for detection specificity is possible environemental change such as temperature. The sensing design strategy using two similar fluorophores as probes which respond to the environmental perturbation in a similar way renders the system to be immune to many potential environmental changes. By taking the ratio of acceptor/donor fluorescence intensities as detection indicator, the donor and acceptor act as internal reference for each other to offset the change of absolute fluorescence intensity change, resulting in a more reliable detection. The data in figure 4 clearly demonstrate that temperature change causes little interference for the detection results.

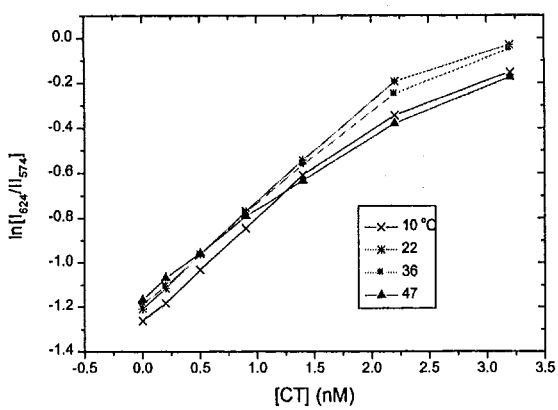


Figure 4: The plots of the fluorescence intensity ratio of B_{TR}-/B_{TMR}-GM1 at different temperatures as a function of CT concentration for samples identical to that used in Figure3

Rapid detection is also crucial for many applications such as medical diagnostics and environmental monitoring. The detection achieved by using energy transfer mechanism is rapid as shown in Figure 5 for the labeled GM1 POPC bilayers on the surface of glass beads. Most of detection can be completed within a few minutes without requiring addition of extra reagents. The detection time depends upon the concentration of both labeled GM1 and CT. As expected, samples with high concentrations of the labeled GM1 respond fast to CT binding.

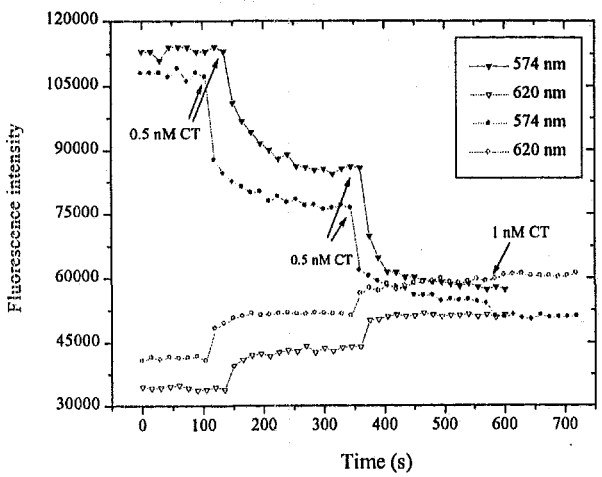


Figure 5: Fluorescence Intensity at 574 nm and 620 nm as a function of time upon addition of CT (ex. at 530 nm) for [B\-(TMR)-GM1]/[B\-(TR)-GM1] =3.5 nM/1.5 nM in the outer leaflet of POPC vesicles (2.5 uM, circle) and [B\-(TMR)-GM1]/[B\-(TR)-GM1 = 2.5 nM/2.5 nM in the outer leaflet of POPC bilayers on glass beads (0.6 mg, triangle)

Aggregation induced energy transfer measured by flow cytometry

The sensing surfaces containing fluid membrane and labeled GM1 can be flexibly constructed on different transduction surfaces. We have demonstrated that such a system can be adapted to waveguide surface for potential sensing miniaturization. A sensitive and convenient platform for the fluorescence measurement on glass microspheres is flow cytometry, which can provide a sensitive fluorescence detection as well as capability of discriminating the fluorescence on the beads from bulk solution. This platform should be very useful for detection of species in a sample containing interfering fluorescent substances. It still provide a low fluorescence background without tedious sample treatment. Figure 6 shows the titration data of the above energy transfer sensing systems obtained from flow cytometer. The fluorescence signal change is proportional to the CT concentration. Similar to the case using fluorimetry, the detection sensitivity and dynamic range are determined by the total amount of the labeled GM in each sample. A low detection sensitivity (c.a 10 pM) can be achieved with in a few minutes. The detailed results obtained from flow cytometry for the energy transfer system will be published in the future.

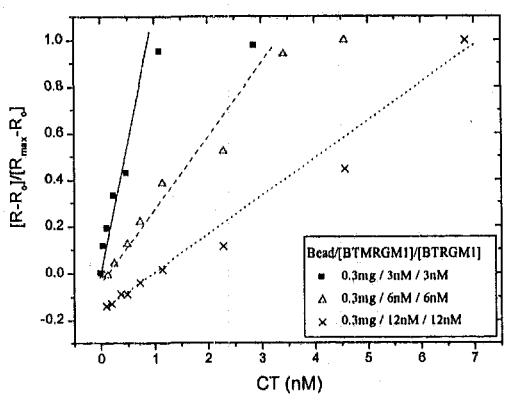


Figure 6: Receptor aggregation induced RET measured by flow cytometry. Microsphere fluorescence was excited at 514 nm with an argon ion laser and donor and acceptor fluorescence was collected through band pass filters and detected with photomultiplier tubes. Data were normalized by subtracting the ratio of samples before addition of CT (Ro), and expressing the result as a fraction of the maximal ratio at saturating CT (Rmax).

Conclusions

The biosensor discussed here by using distance-dependent resonant energy transfer to probe a binding induced receptor aggregation as a signal transduction provides a rapid, highly sensitive and specific method for detection of multivalent interactions. A biomimetic membrane of natural lipids is used to provide a fluid surface for lateral diffusion of the labeled GM1 molecules required for the receptor aggregation. Such a biomimic strategy is very important to improve the effective binding affinity between the receptor and target protein, which limit the detection sensitivity. Since all the sensing elements are integrated into the sensing surface, no extra reagent is required for detection. The method is quite general for multivalent interactions common in biological systems, and should find a number of pharmaceutical and medical applications such as pathogen detection and drug screening. The sensitivity achieved by the method compares favorably with lab-based immunoassay such as enzyme-linked immunosorbant assay (ELISA) method. In contrast to ELISA, the integrated optical biosensor is fast (minutes), simple (one step with no added reagents), insensitive to temperature variation and is robust owing to the stability of the recognition molecules and membranes.

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