Research Paper

A Surface Plasmon Resonance Sensor for Detection of Toluene (C₆H₅CH₃)

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Abstract

Herein, we report a novel surface plasmon resonance (SPR) sensor modified with a toluene-binding domain (TBD) to detect toluene. The TBD was manipulated with cysteines for rapid, easy, and orientation-controlled immobilization of the protein onto an SPR gold surface, due to the specific interaction between bare gold and the thiol group (–SH) of cysteines. After the purification and on-chip immobilization of the recombinant TBD, the thickness of cysteine-tagged TBD was determined to be approximately 1.8 nm according to atomic force microscopic cross-sectional analysis. The SPR analysis of cysteine-tagged TBD adsorption showed that cysteine-tagged TBD binds to gold thin film with high binding affinity. With an SPR-based ligand binding assay, a detectable increase in the SPR response was observed for 15.62 μM of toluene. Owing to the inherent specificity of the TBD, this method showed comparative specificity for the determination of toluene, as it exhibited no SPR response to aromatic compounds such as p-xylene or benzene. Taken together, the results obtained from this study indicate that a TBD-modified SPR sensor system can be useful for detecting toluene, a volatile organic compound widely distributed in the environment.

Keywords: Biosensor, Surface plasmon resonance, SPR, Toluene, Toluene binding domain

1. Introduction

Demand for ligand-binding assays of trace-level environmentally hazardous substances such as aromatic hydrocarbons (e.g., toluene, benzene, xylene), which contain one or more benzene rings, is growing. These volatile organic compounds (VOCs) have been responsible for adverse health effects [1,2]. So far, a variety of techniques have been developed for monitoring the trace levels of chemicals, including solid-phase extraction (SPE) or solid-phase microextraction (SPME) systems with recovery of the pre-concentrated compounds by solvent or thermal desorption [3,4], and qualitative or quantitative analysis of semi-VOCs by gas chromatography (GC) or gas chromatography-mass spectrometry (GC-MS) [5,6]. Although these techniques exhibit high sensitivity and specificity, the approaches require an additional pre-concentration procedure for a long time period to analyze environmental chemicals.

Toluene is a critical ligand required for the activation of TodS/TodT signal transduction in *Pseudomonas putida*

[7,8]. The TodS protein possesses a toluene-binding domain (TBD), which can attach itself to a toluene molecule to trigger autophosphorylation of TodS [9,10]. The TBD of TodS consists of a five-stranded antiparallel β-sheet and three α-helix-spanning residues 45-149, as well as an additional α-helix from residues 150-163 [8]. Toluene binding to the TBD causes conformational changes in TodS [11,12]. With the high and stable interaction between the TBD and toluene, this protein can be potentially exploited as a biological recognition element for detection of toluene.

Surface plasmon resonance (SPR) sensors have been one of the most powerful techniques to determine affinity and specificity during the binding of biomolecules [13]. An SPR sensor does not require any tag or dye for recognizing biomolecules, such as nucleic acids, proteins, peptides, small molecules, or cells [14,15]. As has been extensively and intensively documented in the literature, SPR is an acceptable method for diagnosing infectious diseases, detecting foodborne pathogens, and performing high-throughput screening (HTS) for drug discovery [13-15]. The possible fields of application of SPR technology have expanded to environmental and industrial areas. After the first introduction of a commercial SPR instrument in the

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early 1990s, a variety of SPR-type sensors have been developed for biomolecular interaction studies [13]. Nevertheless, there are very few studies on detecting VOCs with an SPR system. In this study, a novel SPR sensor modified with a TBD as a capture agent was developed to detect toluene.

II. Materials and methods

1. Cloning of cysteine-tagged TBD gene

The forward primer was designed to add an *NdeI* restriction enzyme site, three cysteine residues as a gold binding tag, and a GSGGS linker, while the reversed primer was designed to contain an *XhoI* restriction enzyme site. The PCR product was then purified using a DNA purification kit (Qiagen, CA) and digested with the *NdeI* and *XhoI* restriction enzymes. The Cys:TBD cassette was inserted into the pET-21a (+) vector using *NdeI* and *XhoI* sites to generate in-frame fusion of pCys:TBD. The fusion gene was verified by DNA sequencing and transformed into *Escherichia coli* C41 (DE3) for the expression of the recombinant protein.

2. Expression and purification of the recombinant TBD

The cysteine-tagged TBD construct was transformed into *E. coli* strain DH5a. The ampicillin-resistant transformants were screened. The resulting white colony was inoculated into a 3-ml Luria-Bertani (LB) amp broth culture for an overnight culture at 37 °C. The plasmid was then isolated and purified from cultures using a miniprep DNA purification kit (Qiagen, Germany). The plasmid was transferred to the expression host, the competent *E. coli* strain C41 (DE3) (Stratagene, USA), then plated on LB/ampicillin agar plates. A single colony isolated from a freshly streaked agar plate was picked and grown in a 3-ml LB broth culture with 30 mg/ml ampicillin at 37 °C with vigorous shaking. As soon as an OD₆₀₀ of 0.6 was reached, the culture was inoculated into a 100-ml LB broth culture with ampicillin. The bacterial cells were grown at 37 °C with shaking until the OD₆₀₀ value reached 0.6. The cultures were induced with 1-mM IPTG, grown at 18 °C for 12 h and shaken at 170 rpm. The cultures were then centrifuged at 6000 g for 10 min at 4 °C to harvest the cells. For the purification of the recombinant TBD, the cell lysate was slowly loaded onto a Ni-NTA column, and the column was washed three times with the ice-cold elution buffer (50-mM phosphate, 0.5-N NaCl, pH 8.0) for equilibration. The recombinant TBD was finally eluted with the same elution buffer containing 0.5-M imidazole.

3. SDS-PAGE analysis

The *E. coli* cultures were harvested and suspended in a 50-mM Tris/HCl buffer (pH 8.0) and lysed by three cycles of sonication. The resulting soluble and insoluble fractions were separated using a centrifuge at 10,000 g for 10 min.

The soluble fractions were then subject to a 10 % (v/v) SDS/PAGE analysis to monitor the expression and purification of the recombinant protein.

4. Atomic force microscopy (AFM) analysis

For the AFM analysis, cysteine-tagged TBD (50 µg/ml in a PBS buffer, pH 7.4) was dried on top of a gold chip and scanned with a multimode Nanoscope V controller. AFM images were obtained in the tapping mode under ambient conditions using the Igor Pro 6.36 program. The height and the roughness were determined from horizontal line scans (n = 3 for each crater, 3 craters/sample).

5. SPR measurement

The SPR measurement was performed using a Reichert SPR SR7500DC (Depew, NY). Toluene and other chemicals were dissolved in a PBS buffer containing 1 % DMSO to increase the solubility of the chemicals. The bare gold chip was maintained in a running PBS buffer at a flow rate of 30 µl/min until a stable baseline was achieved. Then, the gold surface was activated with 100 µg/ml of TBD in a PBS buffer for 5 min. After the baseline stabilization, the analytes were injected for 5 min at a flow rate of 30 µl/min. A change in reflectance was detected every second, and the temperature was maintained at 25 °C during the experiment.

III. Results and discussion

1. Principle of SPR biosensor for toluene detection

The aim of this study was to test the ability of a biological recognition element to interact with toluene using an SPR biosensor. Particularly, the TBD of TodS in *Pseudomonas putida* was chosen as the capture agent. The capture protein was modified with three cysteine residues, since the ligand-binding pocket of the TBD might be well exposed when the protein is immobilized on the chip surface via N-terminal cysteine residues. An SPR analysis was performed using the Reichert SPR SR7500DC dual-channel flow spectrometer. A reflectance shift was measured by a photodiode detector, and the data was calculated by Scrubber 2 software. Figure 1 shows the working principle of an SPR sensor system. The SPR-type biosensors have been widely and effectively applied to study interactions for a large range of molecular weights of analytes, from hundreds of Da to the binding of entire cells [16-18]. Since the evanescent field associated with the SPR extends over a length of several hundred nm, the system becomes more sensitive and can be applied to detect the interactions of small molecules in real time. Therefore, the SPR sensor can be a promising vehicle for determining VOCs with high sensitivity and specificity.

2. Expression and purification of cysteine-tagged TBD

His-tag, consisting of six histidine residues, was

Figure 1. Working principle of the SPR sensor system.

Figure 2. Construction of the pET 21a-TBD vector and expression of cysteine-tagged TBD. (a) The map of recombinant plasmid pET 21a-TBD for expression of cysteine-tagged TBD. (b) SDS-PAGE analysis of the recombinant cysteine-tagged TBD.

genetically fused to the C-terminal end of the recombinant protein. This modification facilitates the purification of the capture protein by immobilized metal-ion affinity chromatography (IMAC) [19]. A map of the recombinant pET 21a-TBD expression vector was presented in Fig. 2(a). For the purpose of orientation-controlled immobilization of TBD on the SPR sensor surface, cysteine-mediated immobilization through the formation of the gold-sulfur interaction was performed. The recombinant TBD, modified with three cysteine residues, was expressed in *E. coli* culture, and the expressed protein was purified using an IMAC [Fig. 2(b)]. The purified cysteine-tagged TBD was further applied to the SPR measurement for toluene detection.

3. AFM topography image of TBD layer on a gold surface

AFM analysis was conducted to examine the surface thickness of cysteine-tagged TBD on a gold surface. AFM data can provide information on the homogeneity of TBD immobilized on the surface. The noncontact AFM images

Figure 3. AFM image of cysteine-tagged TBD and the result of cross-sectional analysis. The average height of the TBD layer was approximately 1.8 nm.

of cysteine-tagged TBD surfaces were acquired at a size of 2 µm × 2 µm. Figure 3 shows the AFM three-dimensional image of immobilized-TBD (50 µg/ml) on the SPR sensing layer. The average thickness of the cysteine-tagged TBD layer on the gold surface was calculated to be approximately 1.8 nm, according to the AFM cross-sectional analysis, which is in good agreement with the estimated molecular size of the TBD (43-164 amino acids, ~1.8 nm in length).

4. SPR analysis of cysteine-tagged TBD adsorption

Prior to toluene detection, the adsorption constant of the TBD cysteine-tagged to a bare gold chip was measured using an SPR sensor. Cysteine-tagged TBD binds to the gold surface through the thiol functional group (–SH) of cysteines exposed on the surface of protein. Figure 4 shows the binding affinity of cysteine-tagged TBD. The capture protein was injected over a bare gold chip at different concentrations (i.e., 10, 30, 90, 270, and 810 nM). The sensorgram data are fit to a 1:1 binding model with a mass transport model, and scrubber 2 software was used to fit the data. As shown in Fig. 4, the calculated K_a was $1.81 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, the K_d was $1.89 \times 10^{-5} \text{ s}^{-1}$, and the equilibrium dissociation constant K_D was 104.29 pM, indicating that the cysteine-tagged TBD binds to the gold surface with high binding affinity.

5. Sensitivity for toluene detection

Figure 4. Binding affinity of cysteine-tagged TBD to an SPR gold surface. The sensorgram data are fit to a 1:1 binding model with a mass transport model.

Figure 5. Sensitivity of TBD-modified SPR sensor system. (a) SPR sensorgram of toluene binding at different concentrations of toluene. (b) Calibration curve for toluene.

Figure 6. Specificity of TBD-modified SPR sensor system for toluene over other competing aromatic hydrocarbons. The concentration was 100 μ M for toluene, p-xylene and benzene.

The toluene detection sensitivity of this method was investigated using optimized conditions. For this, a titration experiment was conducted to test whether this sensing system can be used for quantitative detection of toluene. Each solution was injected onto a TBD-immobilized gold surface of SPR with a flow rate of 30 μ L/min. As shown in

Figs. 5(a) and 5(b), the SPR response (uRIU) gradually changed with the increase in toluene concentration. A detectable increase in the SPR response was observed for 15.62 μ M of toluene. The degree of response was well correlated for a toluene concentration in the range of 15.62 to 500 μ M. In the absence of toluene, no SPR response was observed, indicating that the signal was triggered by the presence of toluene. In this work, the detection limit of the system was found to be as high as approximately 15.62 μ M of toluene. Considering that toluene has a lower molecular weight (i.e., 92.14 Da), it is possible that smaller molecules give lower molar responses, leading to relatively poor detection limit.

6. Specificity for toluene detection

For an accurate evaluation of the presence of toluene, a false positive signal with other aromatic hydrocarbons should be tested. Therefore, the specificity for toluene detection was examined by measuring and comparing the SPR response by toluene with that of other chemicals, including p-xylene and benzene, under optimized conditions. As shown in Fig. 6, 100 M of toluene could induce a large enhancement in the SPR response, while other aromatic hydrocarbons resulted in no reflectance changes, or if any, only negligible signals were detected. This result verifies

that the sensing system exhibits high specificity for toluene detection.

IV. Conclusions

The aim of this study was to evaluate the TBD-modified SPR sensor for detecting toluene. The SPR analysis of cysteine-tagged TBD adsorption showed that cysteine-tagged TBD binds to the gold thin film with high binding affinity, as the calculated K_a was $1.81 \text{ e}^5 \text{ M}^{-1} \text{ s}^{-1}$, the K_d was $1.89 \text{ e}^{-5} \text{ s}^{-1}$, and the equilibrium dissociation constant K_D was 104.29 pM. The degree of response was well correlated for a toluene concentration in the range from 15.62 to 500 M. Due to the inherent specificity of TBD, the sensor exhibited excellent specificity for toluene, as no response in reflectance change was observed with other aromatic hydrocarbons, such as p-xylene and benzene. With this effective performance, the TBD-based SPR sensor system can be useful for toluene detection and potentially for monitoring and analysis of environmental pollutants.

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