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A bioinspired peptide matrix for the detection of 2,4,6-trinitrotoluene (TNT)

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Abstract

A novel peptide-based three-dimensional probe called “peptide matrix,” inspired by the antibody paratope region, was fabricated on a surface plasmon resonance (SPR) sensor chip to enhance the sensitivity of detecting the explosive 2,4,6-trinitrotoluene (TNT). Although peptide aptamer is an attractive candidate for a molecular recognition probe because of its ease of synthesis and chemical stability, it still has difficulty in applying to highly sensitive (i.e. parts-per-billion (ppb) or sub-ppb level) detections. Thus, we developed the concept of peptide matrix structure, which is constructed by consecutive disulfide bond formation between a large number of peptide fragments. This robust three-dimensional structure displays multiple binding sites which can efficiently associate with each TNT molecule. The peptide matrix lowered the dissociation constant (K_D) by two orders of magnitude compared to the linear peptide aptamer, estimating K_D as 10.1 nM, which is the lowest concentration reported by using peptide-based TNT probe. Furthermore, the concentration limit of detection of peptide matrix modified SPR sensor was 0.62 ppb, and hence comparable to single-chain variable fragment (scFv)-based TNT sensors. To our knowledge, this is the first report demonstrating peptide matrix fabrication and its application for small explosive molecule detection. This peptide matrix-based approach, which has the advantage of simple synthesis and high sensitivity, will be applicable to many other small-molecule label-free detections.

Keywords

Peptide, Surface plasmon resonance, Explosive detection, Sensor probe

1 Introduction

The trace level detection of explosive compounds has become important for global public security and environmental protection. In particular, 2,4,6-trinitrotoluene (TNT) is one of the most powerful and widely used nitroaromatic compounds for military purposes. TNT is an effective explosive and also toxic to humans and the aquatic ecosystems. Therefore, sensitive and selective TNT detectors are in high demand for aviation security, border control, food safety, and environmental monitoring (Caygill et al., 2012; Lotufo et al., 2010; Rodgers and Bunce, 2001). The conventional sensing approaches include gas-chromatography mass-spectrometry (GC/MS), Fourier-transformed infrared spectroscopy, Raman spectroscopy, and ion-mobility spectrometry (Ewing et al., 2001; Håkansson et al., 2000; Mullen et al., 2006; Pacheco-Londoño et al., 2009; Primera-Pedrozo et al., 2009). Although these techniques have advantages in sensitivity, they have some limitations such as the high cost, requirement of well-trained personnel, and the complexity of sample preparation or calibration.

Label-free detection of nitroaromatic compounds has also been demonstrated using electrochemical sensors, photoluminescence sensors (e.g. quantum dots), and surface plasmon resonance (SPR) sensors (Goldman et al., 2005; Li et al., 2017; Onodera and Toko, 2014; Zhang et al., 2015). In these label-free detection techniques, including SPR, molecular recognition probes having specific and strong binding property to a target molecule are required for achieving reliable detection. However, in general, the fabrication of direct recognition probes to small target molecules such as TNT (MW: 227.1 $g \cdot mol^{-1}$) is challenging. In a previous study, researchers have used pattern analysis technique with macrocyclic molecule immobilized multichannel to identify small explosive analytes (Peveler et al., 2016). Antibodies, or its recombinant proteins, are also used as molecular recognition probes because of their specific and strong binding affinity to analytes (Liu et al., 2013); however, they have crucial drawbacks such as their low stability for long-term storage and the costly and time-consuming production process using animal cells or microorganisms. Other than these, some researchers have taken advantage of molecular imprinted polymer (MIP) integrated with gold nanoparticles (AuNPs) for TNT detection; or the combination of MIP and DNA aptamers for enhancing both the sensitivity and selectivity (Guo et al., 2015; Shahdost-fard and Roushani, 2017). In another case, an anti-TNT peptide aptamer conjugated to AuNPs performed as a molecular recognition element for the graphene oxide-based electrochemiluminescence detection (Yu et al., 2013).

Among these, peptide aptamers are preferable candidates for a sensor probe because of their good chemical stability, ease of synthesis, scale-up, and simple immobilization onto a sensor chip, compared to the protein-based probes (e.g. antibody and receptor molecules). In the previous study, we screened a TNT binding peptide (TNT-BP) from complementarity-determining regions (CDRs) of an anti-TNT antibody (Okochi et al., 2017). Using a peptide array-based TNT binding assay, the 14-mer heavy chain CDR3 (HCDR3; ARGYSSFIYWFFDF) was selected as the strongest binding probe to TNT. The dissociation constant (K_D) of TNT to HCDR3 was determined to be 1.305 μM, by an SPR affinity measurement. Then, HCDR3 was optimized and core 12-mer TNT binding peptide (TNT-BP; ARGYSSFIYWFF) was found

(Okochi et al., 2017). The linear-form TNT-BP has been applied to the SPR sensor devices, and in one case, carbon nanotubes were used to increase peptide probe density on the sensor surface (Wang et al., 2018a, 2018b). Other than this, evolutionary screened TNT binding peptide (WHWQRPLMPVS) was obtained and applied to FET sensor (Justyn W. Jaworski et al., 2008; Kuang et al., 2010). Making use of both peptide probes, the limits of the detection range from 12.9 parts-per-billion (ppb) to 3.4 parts-per-million (ppm) (Table S1). Whereas, it is needed to improve the sensitivity and to achieve ppb/sub-ppb level detection for practical use (e.g. the drinking water standard for a lifetime of exposure to TNT regulated by United States Environmental Protection Agency is 2 ppb) (USEPA, 2018).

Inspired by the recognition site of an antibody, we propose a novel peptide-based three-dimensional "peptide matrix" structure to enhance the affinity of TNT binding peptide probe. In antibodies, six CDRs are composed of amino acid chains of up to several dozen residues to form the specific binding sites for the antigens. There are multiple interactions with the target molecule at the highly-regulated binding pocket using some of six CDRs; this produces the remarkable binding property. The unique structure of peptide matrix is rigidly constructed by multiple TNT-BP fragments and the simultaneous interactions are considered to provide comparable sensitivity to recombinant proteins from antibody (Fig. 1). The peptide matrix is easily fabricated on SPR sensor chip via spontaneous and consecutive disulfide bond formations among cysteines added at both C- and N-terminus end of TNT-BP fragment (Cys-TNT-BP, CARGYSSFIYWFFC). To demonstrate this concept, TNT-BP peptide matrix was assembled on a SPR sensor chip and the affinity and selectivity between TNT (or other analytes) and peptide matrix were evaluated. To the best of our knowledge, this is the first report of a "peptide matrix" probe, which can significantly improve the detection sensitivity of small molecules with SPR and this approach could be applicable to other biosensor devices.

2 Material and methods

2.1 Chemicals and reagents

TentaGel Resin (capacity: 0.25 mmol g^{-1}) was purchased from Intavis AG (Cologne, Germany). N-fluorenyl-9-methoxycarbonyl (Fmoc) protected L-amino acids, *O*-benzotriazole-*N,N,N',N'*-tetra-methyl-uronium-hexafluoro-phosphate (HBTU), diisopropylethylamine (DIEA), trifluoroacetic acid (TFA), triisopropylsilane (TIPS), *N,N*-dimethylformamide (DMF), and 20% piperidine in DMF were purchased from Watanabe Chemical Ind., Ltd. (Hiroshima, Japan). Dichloromethane (DCM), acetic anhydride, ethanol, diethyl ether, acetonitrile, 1-methyl-2-pyrrolidone (NMP), toluene, sodium chloride and phenol were obtained from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan). 1,2-Ethanedithiol (EDT), thioanisole, 2,4-dinitrotoluene (DNT) and amyl nitrate were bought from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). TNT solution, 1,3,5-triethylhexahydro-1,3,5-triazine (RDX) solution (1000 μg mL^{-1} in 1:1 acetonitrile:methanol solution) were purchased from AccuStandard (New Haven ,USA) and 2-propanol (IPA) were purchased from Sigma-Aldrich (St. Louis, USA). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxy succinimide (NHS), 2-(2-pyridinyldithiol) ethaneamine hydrochloride (PDEA), 0.15 M

sodium borate pH8.5 and HBS-EP+ buffer were purchased from GE Healthcare (Chicago, USA). All aqueous solutions were prepared with deionized water.

2.2 Synthesis of Cys-TNT-BP and peptide matrix

Cys-TNT-BP was synthesized following a standard Fmoc-based solid-phase synthesis technique on TentaGel Resin using Respep SL peptide automatic synthesizer (Intavis AG). The cleavage cocktail was mixture of TFA, water, thioanisol, phenol, EDT and TIPS (82.5:5:5:2.5:1). After the peptides were deprotected with piperidine and cleaved from the resin with the cocktail, the collected solution was dissolved in diethyl ether and washed three times. The precipitate was resuspended in 30% acetonitrile and freeze-dried overnight. Finally, the peptides were purified by the ODS-80TS column (Tosoh Corp., Tokyo, Japan) with the HPLC system (LC-20AR, CBM-20A, SIL-20AC, CTO-20AC, SPD-20AV, Shimadzu Corp., Kyoto, Japan). The molecular weights of the peptides were assayed by matrix assisted laser desorption / ionization mass spectrometry (AXIMA-CFRPlus, Shimadzu Corp.) and the final purity of the peptides were determined by ODS-100Z column (Tosoh Corp.) and HPLC (Fig. S1).

To activate the surface of CM5 SPR sensor chip (GE Healthcare), carboxyl groups of carboxymethyl dextran (CM-dextran) were treated by the standard procedure provided by the manufacturer. Purified Cys-TNT-BP was applied to SPR system (Biacore X100 Plus Package, GE Healthcare) to assemble the peptide matrix via disulfide bond formation. Free thiol groups of activated sensor surface and immobilized Cys-TNT-BP were then blocked by adding L-cysteine.

2.3 Matrix fabrication measured by ellipsometry and atomic force microscopy

To observe the peptide matrix assembly, the thicknesses of the matrix on the gold surface was measured at each time point using ellipsometry. A glass slide was cleaned in 10 times diluted Decon 90 liquid detergent and IPA before UV ozone cleaning. The cleaned slide was coated with chromium (5 nm) and gold layer (50 nm) by vacuum deposition. The gold substrate was cleaned up with a UV ozone cleaner and subsequently immersed in IPA for 1 h. The IPA was removed by air blow and 50 μL droplets of the 5 μM Cys-TNT-BP solution were placed on the substrate and each spot was incubated for 5 min, 10 min, 30 min, 1 h, 2 h and 3 h before being washed with deionized water. Three independent spots for each incubation time were measured with M-2000 ellipsometry system (JA Woolham, Lincoln, USA) with the angles of 65°, 70°, and 75°, and evaluated by CompleteEASE software (JA Woolham) assuming a refractive index of 1.45 for the organic film (peptide matrix layer).

In addition, atomic force microscopic (AFM) observation was performed to visually observe the matrix construction. Briefly, a bare gold substrate (Kenis Ltd. Osaka, Japan) was cleaned by CUTE 1MP/R plasma cleaner (International FemtoScience, Inc., Nashville, USA) and then soaked in IPA. The bare gold substrate was measured in MilliQ droplet with NanoWizard ULTRA Speed 2 (Bruker, Massachusetts, USA). Subsequently, 5 μM Cys-TNT-BP and linear TNT-BP-C (ARGYSSFIYWFFC) peptide solution were placed

on the gold substrate. The surface of the substrate was measured during the peptide modification at each time point (15, 30, 45, 60 min). Each measurement was performed by QI mode at the same position on the substrate.

2.4 TNT and TNP-KLH measurements of peptide matrix by SPR

TNT and 2,4,6-trinitrophenyl keyhole limpet hemocyanin (TNP-KLH) conjugate binding affinities of TNT-BP matrix were evaluated by surface plasmon resonance (SPR) system (Biacore X100 Plus Package, GE Healthcare) to evaluate the performance of the molecular capture probe. The Cys-TNT-BP fragments were applied and peptide matrix was fabricated on a CM5 sensor chip by using Thiol Coupling Kit (GE Healthcare). The TNP-KLH conjugate was prepared using a previously reported protocol (Okochi et al., 2017). TNT and TNP-KLH solutions were both diluted with HBS-EP+ buffer in the ranges of 1–81 nM and 0.1–8.1 μM respectively. After injecting five different concentrations of samples with contact and dissociation times of 120 and 600 s, respectively, the response data obtained were analyzed by the Biacore X100 Plus Package evaluation software to estimate the dissociation constants (K_D). Regenerations were performed by 2 M sodium chloride for TNT and 50 mM glycine-HCl (pH 2.0) for TNP-KLH.

2.5 Selectivity assay by SPR

2,4-Dinitrotoluene (DNT) and 1,3,5-trinitro-1,3,5-triazinane (RDX) were selected as nitro explosive compounds. Toluene and amyl nitrate were selected as the representative of aromatic or nitro compounds, respectively. DNT, toluene and amyl nitrate were both diluted with the solvent (1:1 acetonitrile:methanol solution) to 4.4 mM and further diluted to 100 μM with HBS-EP+ buffer. TNT and RDX solutions (4.4 mM in 1:1 acetonitrile:methanol solution) were also diluted to 100 μM with HBS-EP+ buffer. The preparation of the sensor chip is described in section 2.2. Each sample was injected and then regenerations were performed by 2 M sodium chloride after every sample injection. The data was also analyzed by the CompleteEASE software.

3 Results and Discussion

3.1 Fabrication and characterization of peptide matrix

The TNT-BP matrix probe was fabricated on the SPR sensor chip through successive connection of each peptide fragment via spontaneous disulfide bonds. SPR signal during the injection of N- and C-terminus cysteine-modified peptide fragments (960 sec to 1400 sec) constantly increased (i.e. it was unsaturated during the injection period), which indicated that peptide matrix was successfully fabricated on the sensor chip (Fig. S2). Besides, it is suggested that the thickness of the matrix structure is controllable by the peptide injection duration until few free thiol groups remain as connectors between both ends of the polypeptide chain (i.e. end-to-end loop formation).

To determine the duration until saturation of matrix fabrication, the thicknesses of the matrix, as a function of the incubation time, was measured with ellipsometry (Fig. 2). The thickness of the matrix

structure has increasing trend in response to the incubation time, and it reached the plateau at the measuring point of 1 h incubation. Furthermore, AFM observations of TNT-BP linear peptide layer and TNT-BP matrix were performed on the gold substrate (Fig. S3). The root-mean-square (RMS) roughness of bare gold substrate was decreased in response to the incubation time as the peptide layer or matrix were constructed on the substrate, covering the surface ruggedness. Since matrix layer was thicker than linear peptide layer, the roughness of matrix substrate was smaller than that of linear peptide layer, indicating the matrix was formed on the gold substrate.

3.2 The affinity measurement between TNT and peptide matrix

The affinity between TNT and TNT-BP matrix probe was measured to observe the improvement in the efficiency of capturing small target and the results are shown in Fig. 3. The K_D was calculated to be 10.14 nM, which is two orders of magnitude lower than that of the single layer of linear HCDR3 peptide (1.305 μM). The limit of detection (3σ) was also calculated to be 0.62 ppb (2.73 nM), which is lower than that of some reported single-chain variable fragment (scFv)-based TNT sensors (Table S1). Linear dynamic range of this detection system was estimated to be 0.68-18.3 ppb (R^2=0.993), which is comparable to other peptide or recombinant protein-based sensors (Fig.3, Table S1). This significant increase of binding affinity was considered to be achieved by both increase in association and slow apparent dissociation, which are served with the three-dimensional structure of matrix inspired by the antibody CDRs (Lawson et al., 2018). In general, linear peptide probes have lower association activity to target molecule because of its flexibility which prevents stable capture. Thus, some researchers have tackled this problem using a rigid and robust α-helix-based structure as a scaffold (Suzuki and Fujii, 1999). In this study, we mimic the antibody paratope region to realize less structural fluidity and multipoint interactions that provide effective association. Moreover, it is considered that once a TNT molecule dissociates from a binding site of TNT-BP, it is rapidly trapped by another site and this contributes to decreasing the apparent dissociation constant.

3.3 The affinity measurement between large TNT analogue and peptide matrix

To address the hypothesis that the binding pocket size of peptide matrix probe suites only small target molecules, we measured the affinity with the large molecule, TNP-KLH instead of TNT. TNP-KLH consists of 151.5−378.8 trinitrophenyl groups conjugated to the KLH protein (MW: 300−750 kDa) (Okochi et al., 2017). The SPR signal in response to TNP-KLH at each equivalent concentration to TNP is shown in Fig. 4. The evaluated K_D between the peptide matrix and TNP-KLH was 2.08 μM, which is close to that between the linear HCDR3 peptide probe and TNP-KLH (2.01 μM) (Fig. S4). Furthermore, the evaluated affinity of TNT-BP matrix to TNP-KLH is much weaker than that to small TNT molecules (K_D = 10.14 nM). These are consistent with the biomacromolecule not being able to penetrate into the matrix structure, and thus, only interacting with the binding site on the upper surface of the matrix. These findings show that the peptide matrix has a preferable range of analyte size for efficient binding and is especially effective for small

molecules whose molecular weights are less than a few hundred Da.

3.4 Selectivity assay of peptide matrix for the TNT

To investigate the specificity of TNT-BP matrix, a TNT analogue with a smaller number of nitro groups (DNT), two further nitro compounds (RDX and amyl nitrate) and one aromatic non-nitro compound (toluene) were used as analytes and their SPR signals were measured (Fig. 5a). DNT has a similar molecular weight and structure with two nitro groups to TNT. RDX was selected because this is a commonly used nitro explosive and is sometimes mixed with TNT. Toluene was chosen as a control because of its aromatic structure. Amyl nitrate was selected as the representative of non-aromatic nitro compounds. From the results of *in silico* docking simulation between TNT-BP and TNT in previous study, it is predicted that TNT-BP is able to specifically bind to TNT molecules with affinity to both π-electron interaction with the aromatic amino acid residues and electrostatic interaction between the nitro group of TNT and basic amino acid residue Arginine (R) (Dower et al., 1978; Justyn W Jaworski et al., 2008). In this study, the response of TNT is approximately three-times higher than that of RDX, amyl nitrate, and toluene (Fig. 5b). RDX or amyl nitrate with the electrostatic interaction and toluene with π-electron interaction showed lower affinity compared to TNT or DNT which have both of these interactions in its structure. DNT shows slightly lower response compared to TNT because of it's a smaller number of nitro groups. This result indicates the binding between TNT and TNT-BP matrix is attributed to the combination of electrostatic integration and π-electron interaction. In addition, the acetonitrile and methanol solvent, which was diluted to the same concentration to that in the analyte solution indicated poor response of approximately 1 RU. While further optimization of the detection parameters is required, this low reactivity for the organic solvent is preferable for the wipe test of contaminated surface in practical use.

The peptide matrix prepared by consecutive linkage of target molecule affinity peptide via disulfide bonds offers increase in sensitivity to small molecule TNT. This precise molecular recognition probe structure can also be applied to other sensor devices. The tunability of probe thickness is attractive for some sensor devices (e.g. we can easily control the thickness within the Debye length for FET sensor applications). In addition, using a peptide sequence with specific binding ability to a target, it is applicable to sensing variety of small target molecules including explosives, volatile organic compounds as well as disease diagnosis markers. Although it is necessary to optimize the detection conditions for applications using real samples, this novel concept of peptide matrix provides an easy-to-fabricate and cost-effective way to capture small molecules effectively for a wide range of monitoring, sensing, or biomedical diagnosis applications.

Conclusions

We developed a novel concept of assembling peptide matrix inspired from antibodies. This peptide matrix could improve the capturing ability of small molecules due to its rigid matrix structure with multiple binding sites. The matrix form of TNT-BP increased the affinity to small TNT molecules by 100-fold from

its linear peptide form and the limit of detection calculated from SPR measurement was 0.62 ppb, which was similar level to previously reported scFv-based probe. To improve the sensitivity of small molecule detection, peptide matrix fabricated by simple procedure using linear peptide probe may be an attractive sensor interface having multi-binding sites resembling binding pockets of proteins such as antibodies. This technique opens the door for various small molecular detection sensor developments utilized in diverse fields such as environmental monitoring, food quality control, chemical warfare, explosive detection, and health assessment.

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Appendix A. Supplementary data

Supplementary materials to this article can be found in the online version at doi: XXX.

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Figures

Fig. 1 The illustration of TNT-BP matrix via spontaneous disulfide bond generations among Cys-TNT-BP(Not to scale). Small TNT molecules can penetrate into peptide matrix to enhance simultaneous and multipoint interactions between TNT and peptides.

Fig. 2 a The thickness of TNT-BP matrix as measured by ellipsometry. **b** Thickness of matrix on the gold substrate in response to incubation time. Three independent droplets were measured for each incubation time.

Fig. 3 Relative SPR response (R) of the TNT-BP matrix as a function of TNT concentration (C_{TNT}) and the linear dynamic range of the detection in the relationship between $1/C_{TNT}$ and 1/R (inlet).

Fig. 4 a Schematic image of TNP-KLH that cannot penetrate into the peptide matrix structure and interact with the surface region of matrix. **b** The affinity between TNT-BP matrix and TNP conjugated biomacromolecule (TNP-KLH) as shown by SPR signal.

Fig. 5 Specificity analysis of TNT-BP matrix. **a** Structures of TNT analogues. **b** SPR response of TNT (red), RDX (green), amyl nitrate (brown), toluene (blue), and analyte dissolving solvent (gray). A 100 nM of each analyte was injected to the matrix on the SPR sensor chip and the SPR response were measured. Each analyte was applied three times.