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COMMUNICATION

Experimental and Numerical Evaluation of Genetically Engineered M13 Bacteriophage with High Sensitivity and Selectivity for 2,4,6-trinitrotoluene

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Selective and sensitive detection of desired targets are very critical in sensor design. Here, we report an genetically engineered M13 bacteriophage-based sensor system evaluated by quantum mechanics (QM) calculations. Phage display is a facile way to develop desired peptide sequences but the resulting can be imperfect peptide for binding of target molecule. TNT binding peptide(WHW) carrying phage were self-assembled to fabricate thin films and tested for the sensitive and selective surface plasmon resonance based detection of TNT molecules in 500 femto mole level. SPR studies performed with the WHW peptide and control peptides (WAW, WHA, AHW) were well-matched with those of the QM calculations. Our combined method between phage engineering and QM calculation will significantly enhance our ability to design selective and sensitive sensors.

The ability to detect and analyze harmful materials such as explosives[1], pesticides[2], disease markers[3], and food aromas[4] are of importance. Although several highly sensitive sensor systems[5-11] based on oxide layers[12], polymers[13], or even de novo designed receptors[7,14,15] have been developed, lack of selectivity remains one of critical issues, and for this reason many sensors fall short of specifications for many applications. Recently, viruses, which specifically infect a target host cell, have attracted the attentions of those trying to develop highly selective and sensitive sensors[16,17]. Although the natural function of viruses is to store and transport genetic materials, they have recently been demonstrated as templates for the synthesis and assembly of functional nanomaterials and devices[18-25]. Specifically, the incorporation of specific binding peptides, identified by evolutionary screening process called phage display, have been used to enhance the specificity of sensor systems[17,26-28]. Among various viruses, M13 bacteriophage (M13 phage), a filamentous bacterial virus, has been extensively investigated. Because of its high aspect ratio (880nm in length, 6.6nm in width), M13 phage exhibits excellent self-assembly behaviors similar to that of liquid crystal molecules[29-31]. Phage display has been widely utilized to modify the surface properties of M13 phage such that it displays desired functional peptides on its surface proteins[32]. The phage display processes comprise of multiple procedures including target exposure, washing, elution, and amplification process. Using phage display process, specific peptides for the desired target materials are isolated. After the isolation of the peptides, it is required to confirm their binding affinity and specificity for the target through the repetitive and labor intensive binding assays. Various of binding assays including comparative phage binding assays, ELISA, spectroscopy, surface plasmon resonance, nuclear magnetic resonance have been utilized to confirm the binding affinity and specificity. Furthermore, because phage display is based on affinity binding and amplification, phage display may result in false-positive and non-specific binding peptides. Therefore, conventional phage display process and binding assays are burdensome process although microfluidic approaches has been implemented[33, 34].

In this study, quantum mechanics (QM) calculation was introduced to compensate the vulnerability of typical phage display techniques. We developed binding characterization method between the consensus peptides and target molecules exploiting QM calculation to characterize the binding specificity between isolated peptides and target molecules. In order to determine whether QM calculations provide an appropriate means of identifying best peptide binding sequences for target molecules, comprehensive studies have been performed a genetic construction of engineered phages with model

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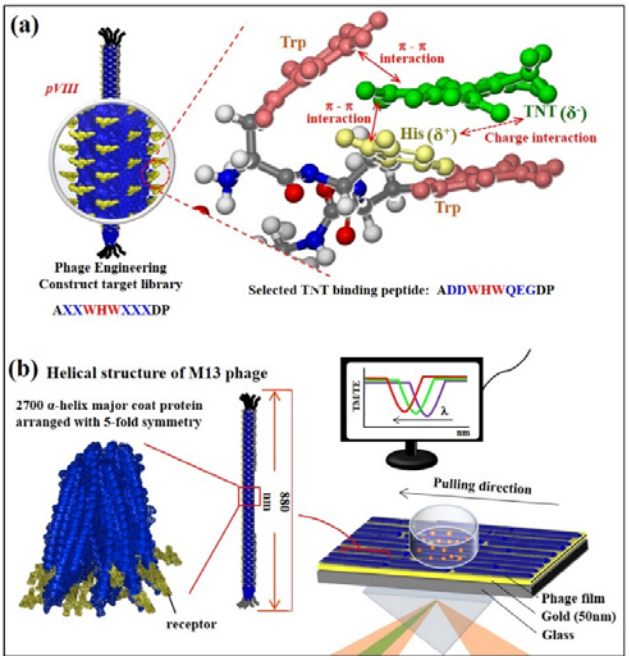
peptides, self-assembly of the phage films, SPR characterization, and QM calculation. Our resulting SPR studies based on the phages with model peptides provides a straightforward way of confirming QM calculation results because of its extreme sensitivity to surface dielectric properties.

We utilized a phage-based model system to study the interaction between 2,4,6-trinitrotoluene (TNT) target molecules and a TNT binding peptide[1,26,37] (Scheme 1a). In order to develop major coat protein engineered phage for TNT molecules, we previously identified the TNT binding peptide using phage display with a commercially available 12mer linear peptide library (Ph.D.™-12, New England Biolab, Ipswich, MA).1 We identified the consensus TNT binding peptide (Tryptophan(Trp)-Histidine(His)-Trp: WHW) and confirmed it's specificity.[1] We also incorporated the consensus TNT binding peptide (WHW) on the major coat proteins (pVIII) of the M13 phages.[19] In order to compare the specificity of WHW engineered phage, we constructed alanine(Ala)-substituted control phage (WAW, AHW, WHA) using site-directed mutagenesis of the WHW-phage. The constructed phages were amplified using bacterial cultures and purified through standard polyethylene glycol precipitation. The phage solution was further purified by filtration through 0.45 μm pore size membranes. To verify phage stability, DNA sequences were confirmed at each step of the amplification at the DNA Sequencing Facility at University of California, Berkeley (Berkeley, CA).

The WHW peptides possess an interesting molecular structure to recognize the TNT target. The π electron-rich group of WHW peptide can bind TNT through the π-π interaction. In addition, partial negative charge of the three nitro-groups in the TNT can



interact with the partial positive group of the imidazole of the His in the WHW peptides. In order to investigate the specific interaction between TNT molecules and WHW peptides using a surface plasmon resonance (SPR) spectroscopy, we fabricated

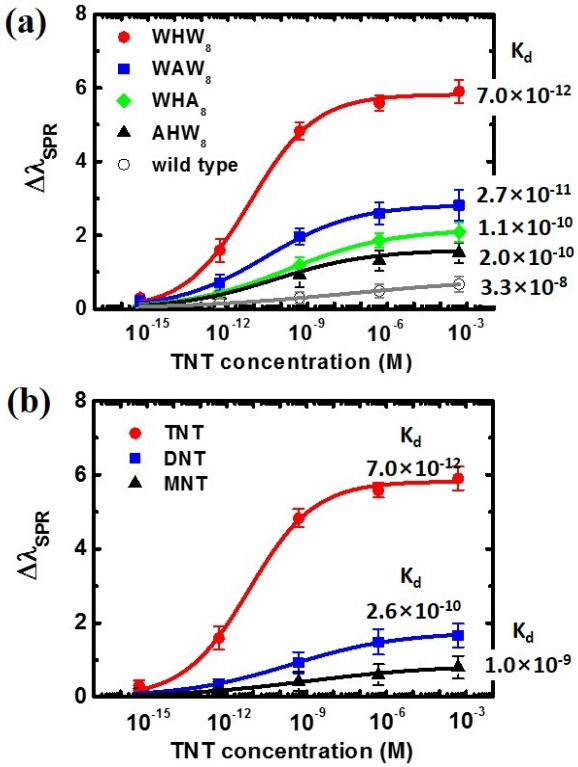


Figure 1 (a) SPR wavelength shift of WHW, WAW, WHA, AHW-phage-based SPR sensors under the various concentration of TNT exposure (b) SPR wavelength shift of WHW-phage-based SPR sensors under the various concentration of TNT, DNT and MNT exposure.

self-assembled TNT sensor matrices composed of WHW-engineered M13 phage (WHW-phage) using a self-templating process[29, 38] (Figure S2). Each phage displays ~2700 copies of the WHW peptide on the surface of the phage. Using the self-templating process, we can fabricate self-assembled phage films which can exhibit high density of the recognition peptides (Scheme 1b). AFM analysis of self-assembled structure showed that WHW-phage formed directionally aligned liquid crystalline film (Figure S3) (pulling speed: 300μm/min). In order to study the specificity of the phage, we also constructed the alanine substituted phage (AHW-, WAW-, WHA-phage) and fabricated the liquid crystalline phage films using a similar approach. We also fabricate a wild type phage films with the similar liquid crystalline morphology (Figure S4). Compared with wild type phage films, WHW-type phage and its alanine substituted phages assembled into bundle structure. This is because of the π-π interaction between tryptophan or histidine-containing phage39. Such nematic arrangements of dielectric materials are known to substantially enhance surface plasmon resonance (SPR) [40,41].

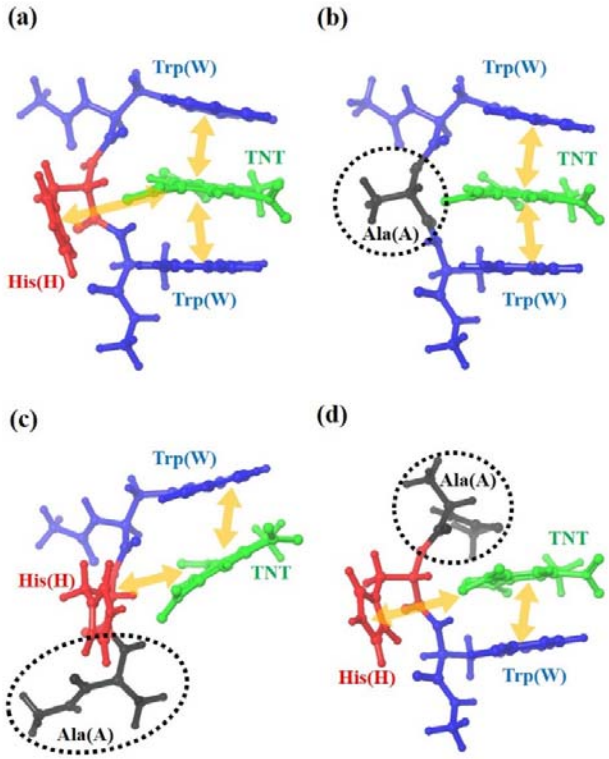


Figure 3 Most stable conformations of TNT-peptides (Red : Histidine, Blue : Tryptophan, Black : Alanine, Green : TNT, Orange : π-π interaction) . (a) TNT-WHW, (b) TNT-WAW, (c) TNT-WHA, and (d) TNT-AHW.

We characterize the interaction between WHW-phage and other control phage film with TNT target molecules using SPR. We monitored real time SPR wavelength shifts ($\Delta\lambda_{SPR}$) obtained by reflectance (TM/TE) upon the addition of analytes at concentrations between 500 aM to 500 μM in ethanol. The WHW-phage film exhibited that the λ_{SPR} shift of the SPR sensor showed significant increases to TNT concentration increases from 500 fM to 500 nM and gradual saturation after 500 μM (Figure 1A). The WHW-phage SPR sensor allowed TNT detection at concentration down to 500 fM, which is markedly lower than those reported previously for TNT sensor systems using different transducers[19, 42-45]. Binding constant (K_d) of the WHW-phage for the TNT is 7.0x 10^{-12}M. This observation indicates that the addition of TNT induced a shift in the refractive index of WHW-phage matrices by binding to WHW, which agrees well with our previous results[19,27,31,46]. To further examine the interaction between WHW-phage and TNT, control binding assays were performed using WHW-phage, alanine-substituted phages, and wild type phage. As shown in Figure 1A, alanine-substituted phages and wild-type phage exhibited markedly lower binding affinities. Binding constants (K_d) for WAW-, WHA-, AHW-, and wild-type phage for the TNT molecules are 2.7 x10^{-11}, 1.1 x 10^{-10}, 2.0 x10^{-10}, and 3.3 x 10^{-8}M. Substitution of His with Ala showed bigger affinity change between WHW-peptide and TNT molecules than that of Trp. Therefore, both electrostatic

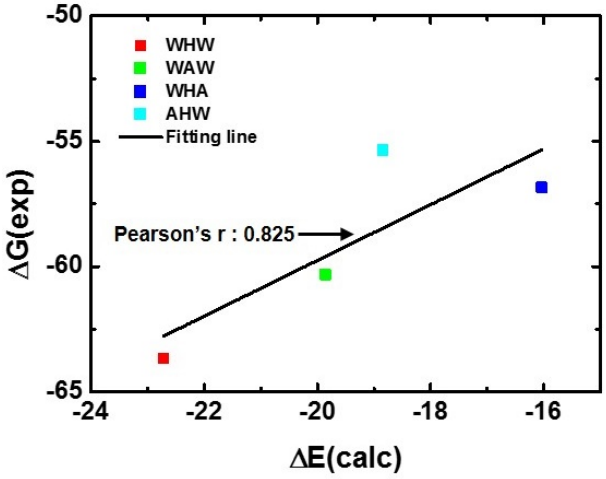


Figure 2 Comparison between calculated values, ΔE(calc), and experimental values, ΔG(exp) plotted in square dots. The result of linear fitting and pearson's correlation coefficient is also displayed respectively.

interaction and hydrophobic pi-pi interaction play a critical role in the recognition of WHW-peptide and TNT.

In order to investigate the specificity, we carried out control experiments using the aromatic compounds dinitrotoluene(DNT) and mononitrotoluene(MNT), which have molecular structures similar to that of TNT. The additions of DNT or MNT also caused λ_{SPR} increases of WHW-phage based SPR sensors, but their sensitivities were significantly lower than that of TNT. The affinity of WHW-phage film with DNT was 2.6 x10^{-10}M, and that of MNT was 2.0 x10^{-10} M (Figure 1B). The SPR results are well matched with QM calculation and demonstrated the high sensitivity and selectivity of the WHW-phage based SPR sensor. We investigated the WHW-peptide and their interaction with TNT molecule using QM calculation. QM calculations were used to determine whether WHW-phage is most specific functional M13 phage for TNT. All QM calculations were performed using Jaguar v8.4 software. To obtain the geometries and energies of various molecules, we used the M06-2X/6-31G** level of density functional theory (DFT) calculations. The tripeptide, WHW and its Ala-substituted peptides, WAW, WHA, and AHW were used to compute TNT binding properties. In order to minimize the perturbation from the N- and C-terminal functional groups, N-terminals of these peptides were acetylated, C-terminals were terminated with N-methylamide, and the imidazole ring of His residues were protonated to reflect a low pH environment. In order to generate several peptide conformers, initial values of Ramachandran angles corresponding to the various secondary structures were chosen, and the resultant structures were then optimized. Extended conformations were found to be most stable for free, unbound tripeptides except AHW, for which a left-handed alpha helical form was most stable. Supporting information (Figure S5) provides the most stable conformations of the four peptides. When binding TNT, all three residues of WHW tripeptide are involved (Figure 2a). Two tryptophan rings bound to the phenyl ring of TNT due to π-π interactions, and the

protonated imidazole ring bound to a NO_2 of TNT due to π and electrostatic interactions. The binding energy of WHW-TNT was calculated to be 22.7 kcal/mol. For WAW, the methyl side chain of Ala did not contribute to TNT binding, rather binding involved two π-π interactions (Figure 2b). The binding energy of WAW was calculated to be 19.9 kcal/mol. For WHA and AHW, each Trp and His contributed to TNT binding (Figures 3c and 3d), and these binding energies were calculated to be 16.0 and 18.8 kcal/mol, respectively. Table 1 shows calculated binding energy values (ΔE(calc)), Gibbs free energy values (ΔG(exp)), and experimentally obtained K_d values for TNT. ΔG(exp) values were derived from experimental K_d values. To demonstrate the correlation between experimental and calculated results, linear fitting was performed (Figure 3). Pearson correlation coefficient (r) was determined to 0.825 indicating a strong linear relationship between ΔE(calc) and ΔG(exp).

Table 1. Calculated and experimentally derived values.

Sequence	ΔE(calc) [a]	ΔG(exp)[b]	K_d[c]
WHW	-22.7	-63.7	7.00E-12
WAW	-19.9	-60.3	2.70E-11
WHA	-16.0	-56.8	1.10E-10
AHW	-18.8	-55.4	2.00E-10

[a] Binding energy values between tripeptides and TNT obtained by QM calculation. [b] Gibbs free energy values for interactions between tripeptides and TNT derived from experimental K_d values. [c] Dissociation constants derived from experimental results.

Conclusions

In this study, we fabricated phage-based sensing matrices and studied their interaction with the target molecules using SPR. We also used QM calculation to study the most stable binding conformation and their binding energy. Both SPR and QM calculation results showed that WHW can recognize the TNT target molecules in a highly sensitive and selective manner. The binding mechanism of the WHW peptide against TNT was verified using QM calculation. There are two key interactions. One is aromatic-aromatic interactions between aromatic structures of Trp and TNT. The other is partial positive charge of the nitrogen in imidazole ring and partial negative charges of oxygen in nitro group of the TNT. Through the multivalent interactions between TNT and WHW peptides, the specific recognition was achieved, which was confirmed in SPR results. Mutation of indole group or imidazole group in WHW peptide significantly reduced the binding affinity to the target. In addition, the MNT and DNT binding affinity to the WHW peptides was significantly reduced. Our combined approaches of phage engineering and QM computation will significantly enhance our ability to characterize the binding peptides and target molecules interaction in the future.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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