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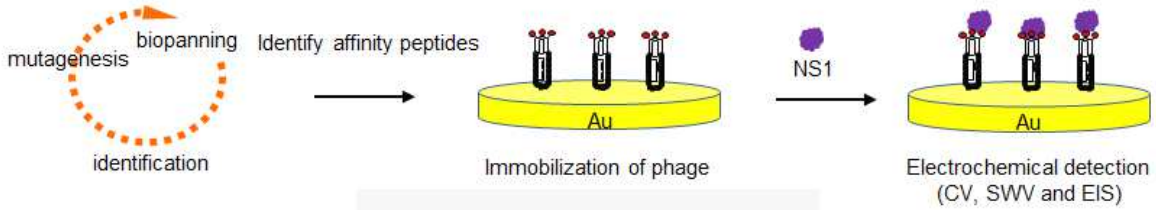

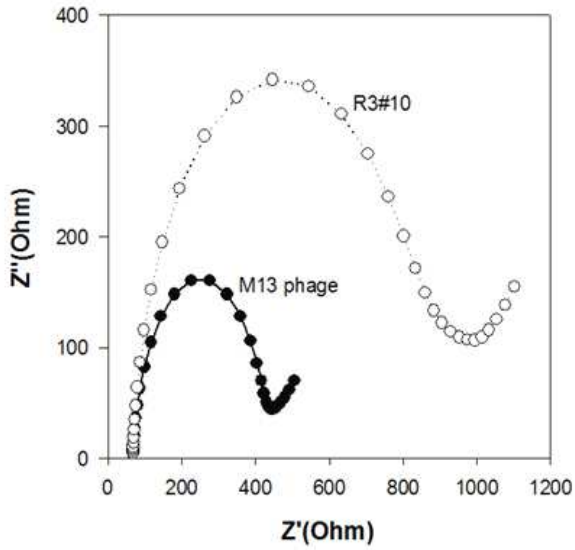

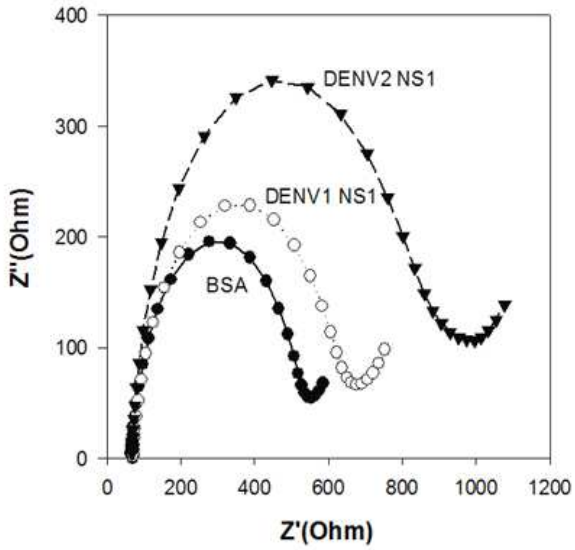

An electrochemical peptide sensor for detection of dengue fever biomarker NS1

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Notes:

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ABSTRACT

Dengue virus type 2 NS1 (DENV2 NS1) is a specific and sensitive protein biomarker for dengue fever diagnosis. In this study we used polyvalent phage display to identify unique affinity peptides that can bind NS1 protein. Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were used to investigate the binding interactions. The potential affinity peptide-displayed phage from these methods was selected; its sequence was EHDRMHAYYLTR (R3#10). Amino acid sequence analysis showed that the peptide was rich in basic residues (two His and Arg). Among all the peptides tested, R3#10 showed the greatest decrease in current in CV and increase in impedance in EIS upon binding to NS1 proteins. EIS revealed that R3#10 phage clones were more specific towards NS1 proteins, as compared to bovine serum albumin or the M13 wild type used as control. Detection of NS1 proteins is in accordance with the electron-transfer resistance (R_{ct}) value of the sensor layer, which is confirmed by EIS, and the K_d value of the R3#10 peptide while binding to the phage particles was measured. To the best of our knowledge, this is the first example of identification and characterization of NS1 binding affinity peptides using phage display technology and electrochemical methods. We concluded that these new peptide-displayed phages or free peptides from phages may have potential applications in dengue diagnosis.

Keywords: Dengue virus, early diagnosis, affinity peptide, electrochemical detection, biosensor

1. Introduction

Dengue fever is one of the most acute mosquito-transmitted viral infections in the world. Dengue virus infection can cause dengue fever, dengue hemorrhagic fever, and dengue shock syndrome. Globally, 2.5 billion people are estimated to be at high risk of infection, with 50 million infections occurring each year, resulting in 500,000 cases of dengue hemorrhagic fever and at least 22,000 deaths. There are 4 distinct types of dengue viruses (1–4), and it is genetically related to other flaviviruses. Dengue virus (family of Flaviviridae) is relatively small (40–60 nm) with spherical particles and an isometric nucleocapsid [1]. Their genome is composed of a single RNA (~11 kb), which encodes three structural and seven non-structural proteins [1]. The gold standard for detection of dengue virus is the direct isolation of virus from cell culture [2, 3]. However, this method is time-consuming, labor intensive, and requires weeks to deliver precise outcomes. Accurate and cost-effective diagnostic assays are highly desirable for clinical diagnosis. Some reports suggest that the NS1 protein is a useful dengue fever biomarker because of its release form infected cells into the bloodstream and can be accumulated in dengue patients at concentrations up to 50 μg/mL [2].

Detection of specific viral particles and serological detection with specific anti-dengue antibodies are commonly used alternatives for rapid diagnosis of dengue virus infections [3-5]. Serological tests identify IgM and IgG antibodies against the dengue virus [5]. However, these methods are unreliable and inaccurate in some cases because of cross-reactivity and low sensitivity. To address these limitations, alternative diagnostic methods, such as RT-PCR [2, 6], nucleic acid-based assay [7-9], nanomaterials-based assay [10-12], miniaturized micro-system- or bead-based assay [13-15], and other assays [16-19] have been demonstrated. For

example, RT-PCR products from dengue viruses (type 1 or 2) were detected using silicon nanowire-based biosensor or nanostructured membrane. Although the method was sensitive, these methods require multiple steps (incubation and/or washing process) for sample preparation, thus increasing the assay cost, assay time, and complexity. Therefore, simple and rapid diagnostic systems with high performing sensors have attracted tremendous attention.

A number of electrochemical detection platforms have been developed [15, 20, 21]. Among the various biosensing methods, cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS)-based detection methods have been widely used because of their rapid response, reliability, and label-free detection [22, 23]. Both methods show ultrasensitive levels of detection of the desired analytes.

As described in previous studies, current immunoassay relies mainly on antibodies as affinity reagents, and is relatively expensive for multiple-sample preparation [22, 24, 25]. Moreover, it is also prone to false-positive outcomes. Therefore, linear and unique short peptides are becoming increasingly popular as effective affinity reagents in the development of new biosensors [25-27]. Their small sizes and cost efficiencies make them a lucrative option for creation of a new biosensing platform [22, 27]. Generally, peptides exist in linear or cylic forms, indicating that peptides are more amenable than antibodies to engineer at the molecular level. In addition, peptides can easily attach to the surfaces of different materials, such as nanoparticles or liposomes for bioimaging and bioassays. Phage-display technique is a widely used method for identifying unique affinity peptides that selectively bind several targets [22, 28]. This procedure consisted of the following steps: 1) phages displaying peptide interact with the chosen targets and unbound phages were washed away; 2) bound phages were eluted using harsh acidic pH conditions (in pH 2.2), neutralized, and amplified in *E. coli*;

and 3) individual phage clones were sequenced and phage-ELISA was performed. There have been some reports using entire phage clones or free peptides from these selections as recognition elements in various types of biosensors. Using this approach, our group also recently reported a new biosensor for detecting troponin I (acute myocardial infarction biomarker) [29], noroviral protein [27] or norovirus [22], procalcitonin (human sepsis biomarker) [26], alanine aminotransferase (human liver function biomarker) [23], and colon cancer biomarker [24]. It is well-known that the interactions of the individual affinity peptides with their corresponding target proteins may be weak. However, the interactions can be stronger when multiple copies of affinity peptides are displayed on the surface of phage particles [29]. For such reasons, many researchers have demonstrated the use of whole unmodified phage particles for developing new biosensor.

In this study, we used the polyvalent phage-display system to identify novel sequences that are capable of binding to dengue fever biomarker NS1 proteins. High affinity peptides were identified from biopanning, and their functional affinity was characterized using CV and EIS. To the best of our knowledge, this is the first study showing discovery and characterization of newly identified affinity peptide binders for detection of dengue fever biomarker NS1.

2. Experimental Section

2.1. Chemicals

Bovine serum albumin (BSA) was purchased from Sigma-Aldrich (St. Louis, MO). Recombinant dengue virus type 1 NS1 proteins (DENV1 NS1, ~40 kDa, >90% purity)) and type 2 NS1 proteins (DENV2 NS1, ~48 kDa, >90% purity) were purchased from Abcam

(Cambridge, MA). Phosphate buffered saline (PBS, pH 7.0) and Tris-HCl buffer solutions (pH 7.2) were used for all protein preparations or samples for CV and EIS measurements. Unless otherwise stated, other reagents were analytical grade.

2.2. E. coli strains and bacteriophages

Escherichia coli strain ER2738 [F'*proA*$^{+}$*B*$^{+}$ *lacI*^{q} Δ(*lacZ*)*M15 zzf::Tn*10(Tet^{R})/*fhuA2 glnV* Δ*(lac-proAB) thi-1* Δ*(hsdSmcrB)5*] that was used as host for M13 phage infection and M13 random peptide library (Ph.D.-12) that expresses random and linear 12-mer peptides were obtained from New England Biolabs (Ipswich, MA). In brief, this random peptide library was fused to pIII protein, resulting in five copies of a particular peptide to be displayed on the surface of phage, and the sequence of each peptide was encoded in the phage genome.

2.3. General M13 methods and DNA sequencing analysis

Phage purification, concentration, and DNA isolation were carried out according to instructions of the manufacturer. Clones of interest were sequenced using the -96 pIII sequencing primer (New England Biolabs, 5'-GCCCTCATAGTTAGCGTAACG-3'), as previously reported [27, 29] .

2.4. Biotin labeling of recombinant dengue virus NS1 proteins

Biotinylation of the recombinant DENV2 NS1 proteins was carried out according to instructions of the manufacturer (Pierce Biotechnology, Rockford, IL). Briefly, NS1 protein (50 μg) was mixed with 10 mM of biotin solution (20-fold molar excess) and incubated on ice for 3 h. To remove non-reacted and hydrolyzed biotinylation reagents, reaction mixtures

were purified using a Zeba desalting spin column (Thermo Fisher Scientific, Waltham, MA). To estimate the level of biotin incorporation, a solution containing biotinylated NS1 was added to a HABA (4'-hydroxyazobenzene-2-carboxylic acid)/avidin solution, and absorbance of this solution at 500 nm was used to calculate the number of moles of biotin per mole of protein.

2.5. Biopanning phage-displayed peptides

Recombinant DENV2 NS1 proteins were dissolved in 50 mM PBS buffer (pH 7) and transferred to wells of the 96-microwell plates. After overnight incubation at 4 ℃ with mild shaking, the coated wells were filled with blocking buffer (0.1 M $NaHCO_3$ (pH 8.6), 5 mg/mL BSA, 0.02 % NaN_3) and incubated at 4 °C for 1 h. After removing the blocking solution, the pre-coated wells were washed six times with TBST (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20). The Ph.D.-12 peptide library (1.5×10^{13} pfu) in 100 μL of TBS buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl) was added to each well containing immobilized recombinant NS1 proteins, and the plate was incubated overnight at 4 °C with gently shaking. To remove unbound phages, the plate was washed 10 times with TBST. Biopanning experiments were performed for the NS1 protein. After washing, the bound phages were eluted using 100 μL of 0.2 M glycine–HCl (pH 2.2). The eluent was immediately neutralized with 15 μL Tris–HCl (pH 9.1) to prevent destruction of the phage. The eluted phages were amplified using *E. coli* ER2738 strains to make sufficient copies for subsequent rounds of biopanning. The amplified phages were harvested by NaCl/polyethylene glycol precipitation (20% (v/v) PEG-8000 with 2.5 M NaCl). After every round of biopanning, the recovered phages were titered by plating aliquots of the infected *E.*

coli ER2738 prior to amplification on Luria–Bertani (LB) agar containing isopropyl β-D-thiogalactopyranoside (IPTG) and X-gal. The plates were incubated overnight at 37 °C, and the blue colonies were counted. Enrichment of bound phages was calculated as follows: output phage/input phage × 100. The blue plaques were randomly picked and amplified for DNA sequencing.

2.6. Sequence analysis

Single-stranded DNA strands from positive phage clones were sequenced by Genotech (Daejeon, Korea) using the -96 pIII sequencing primer (5′-CCC TCA TAG TTA GCG TAA CG-3′). Based on the phage pIII gene-derived reading frame in the coding strand, we derived the amino acid sequence of the exogenous protein fused with the M13 coat protein pIII. BLAST searches were performed using the SWISSPROT database to determine sequence similarity with previously identified human peptides or proteins. The Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/) and ExPASy Tools (web.*expasy*.org/*translate*) were used to analyze the sequences identified earlier.

2.7. Measurement of equilibrium dissociation constants

Equilibrium dissociation constants (K_d) for the selected peptides were measured from the change of electron-transfer resistance (R_{ct}) with a range of DENV2 NS1 protein concentration in EIS. Briefly, gold sensor electrodes were coated with DENV2 NS1 proteins at various concentrations (0.025–12.5 μg/mL) for 1 h at 4 °C and washed six times with PBS solution. Then, the desired R3#10 phage particles (about 10^9 pfu/mL) in PBS buffer were added and incubated for 1 h at room temperature. After incubation, the gold electrode was washed six times with PBS solution. The bound peptide-displayed phages were detected by change in R_{ct}

in EIS. The equilibrium dissociation constants (K_d values) were estimated from slopes of the regression curves obtained by plotting the R_{ct} value upon binding peptide-displayed phage versus the molar concentration of DENV2 NS1 protein.

2.8. Cyclic voltammetry (CV), square wave voltammetry (SWV) and electrochemical impedance spectroscopy *(EIS) measurements*

The conventionally used three-electrodes, working electrode, platinum counter electrode, and Ag/AgCl reference electrode, were used for electrochemical measurements. CV and EIS were performed using an electrochemical analyzer (CHI 750E, CH Instruments, Austin, TX) connected to a computer data analysis system. These analyses were conducted in a PBS solution with 4 mM of ferro/ferricyanide, as described in our previous study [22]. CV measurements were recorded from 0.0 V to +0.4 V vs. Ag/AgCl electrode at a sweep rate of 20 mV/s. Square wave voltammograms were carried out in the range of -400 to 800 mV with a step potential of 4 mV, amplitude of 5 mV and frequency of 10 Hz. EIS was carried out at a formal DC potential of 0.2 V using an alternating voltage of 10 mV in the frequency range from 10 kHz to 10 Hz.

2.9. Statistical Analysis

Two-way ANOVA was used to determine significance of the binding affinity peptides (GraphPad Software Inc, La Jolla, CA). Student's t-test was used to compare the ΔR_{ct} values.

3. Results and Discussion

3.1. Identification of DENV2 NS1 glycoprotein binding phage particles

In this study, DENV2 NS1 protein was used as a target for selecting binding affinity

peptides in the M13 phage-display technique. Antibody-based immunoassay has been developed for diagnosis of DENV infections [30]. However, IgM tests are not reliably positive and specific for acute DENV because of cross-reactivity. In addition, IgG does not distinguish current symptoms from those from a past infection. Non-structural glycoprotein (NS)-antigen detection is more specific, and its sensitivity is lower, as compared with primary dengue infection. NS1 is secreted in DENV-infected mammalian cells and released in their blood stream [30]. Thus, it can be detected in the plasma of the patient because the amount of NS1 in human sera appears to be significantly higher in patients. Therefore, NS1 proteins may have great potential as biomarker antigens to detect dengue virus. To the best of our knowledge, binding peptides specific for DENV2 NS1 have not been reported.

To identify affinity peptides that bind dengue NS1, we biopanned an M13 random peptide library for DENV2 NS1 protein. Briefly, biotinylated recombinant DENV2 NS1 proteins were immobilized onto streptavidin-coated micro-well plates and washed with TBST after blocking. The Ph.D.-12 phage-displayed random peptide library (1.5×10^{13} pfu) was added into the pre-coated micro-well plate, and the bound phages were eluted. After 3 rounds of biopanning, the affinity peptides that were specific for NS1 were identified. The percent yields after 3 rounds biopanning are shown in Table S1. The enrichment of the eluted phage clones increased with increasing number of biopanning, as expected. Samples of the eluted phages from second and third rounds of biopanning were used to purify individual phages, and 30 of these were analyzed by DNA sequencing. During the biopanning process, we chose five potential candidates capable of binding to DENV2 NS1 (R2#2 with amino acid sequence IPLLNPGSMQLS, R2#4 with amino acid sequence SPPWLPVMPVRA, R2#9 with amino acid sequence WHYNWQDVSDRQ, R2#10 with amino acid sequence

HSYTQTSLWMKV, and R3#10 with amino acid sequence EHDRMHAYYLTR). Thereafter, relative binding affinity for NS1 was measured by CV and EIS. The amino acid sequence of the selected peptide-displayed phages is shown in Table 1.

3.2. Sequence analysis of isolated peptide-displayed phage clones

In order to study sequence-based binding affinity, amino acid sequences of the selected phage clones were analyzed using the program PEPTIDE 2.0 (http://peptide2.com/N_peptide_hydrophobicity_hydrophilicity.php). R3#10 phage clones were richer in basic residues (e.g., two His and two Arg), as compared with other 3 phage clones. The total number of basic residues in R3#10 was slightly higher than those in other phages. Interestingly, R2#4 phage clones were rich in hydrophobic uncharged residues (e.g., four Pro, two Val, one Trp, Met, Leu, and Ala), and hydrophobicity of R2#4 phage was the highest. In addition, there was no acidic residue in R2#2, R2#4, and R2#10 phage clones (Table 1). Thus, the allowance of basic amino acid residues (two His and Arg) in peptide sequence may be dominantly involved in binding to DENV2 NS1.

3.3. Characterization of the selected phage clones by electrochemical detection (SWV and EIS)

Fig. 1 shows relative binding affinities of selected five phage clones upon binding to DENV2 NS1 by measuring in SWV. It was observed that the binding interaction between peptide-displayed phage clones and DENV2 NS1 proteins causes a decrease in the current intensity in SWV. Among the five phage clones tested, R3#10 showed the highest binding affinity to NS1. The decrease in current with R3#10 phage particles was the most significant, indicating that specific binding of DENV2 NS1 proteins with R3#10 peptide-displayed

phages occurred on the gold sensor layer. The order of current change was as follows: R3#10 > R2#9 > R2#2, R2#4, and R2#10. However, binding affinities of R2#2, R2#4, and R2#10 phage clones were relatively low, indicating lower binding affinities for their targets. This fact was in agreement with the results obtained from EIS measurements (Fig. 2). A dynamic response was observed with R3#10 phage clones in EIS. An EIS spectrum is commonly represented by a Nyquist plot with Z' versus Z'', where Z' and Z'' are real and imaginary parts, respectively. This means that a larger semicircle indicates a larger value of Z''. Thus, relative impedance increased with increased binding of DENV2 NS1 to the peptide-displayed phage particles. R3#10 phage particles showed the highest increase in impedance. However, no significant changes were observed when R2#2, R2#4, R2#9, and R2#10 were applied. Based on these results, we selected R3#10 peptide-displayed phages as the potential affinity peptide for detection of DENV2 NS1 proteins.

3.4. Effects of DENV2 NS1 protein and peptide-displayed phage concentration on binding interactions

In order to investigate the concentration of DENV2 NS1 protein on binding of peptides, protein concentrations were varied from 1.5 μg/mL to 25 μg/mL, and the change in impedance in EIS (Fig. 3a and 3b) and the change in current in SWV (Fig. 3c and 3d) were monitored. As shown in Fig. 3a and 3c, quantitative changes in the impedance and current intensity were observed with increasing DENV2 NS1 protein concentrations. To verify the effects of peptide-displayed phage concentration on binding interactions, different concentrations of R3#10 phages (10^5 to 10^9 pfu/mL) were incubated at 20 μg/mL of DENV2 NS1 proteins and the impedance change in EIS and the current intensity in SWV were

measured. Significant impedance changes and decrease of current were observed in a concentration-dependent manner (Fig. 3b and 3d). Thus, we concluded that R3#10 phage particles specifically detect DENV2 NS1 proteins.

3.5. Effect of serum on binding interactions

The measurement of fever biomarker NS1 in cell culture environment or liquid biopsy in human may be important for dengue diagnosis. Therefore, we tested the effect of serum on the binding of NS1 proteins. A total of 10^9 PFU/mL of R3#10 phage clones were incubated in the presence of serum (0-0.5%) at room temperature for 1 hr and the binding affinity was measured by EIS. We observed that addition of serum slightly affected the binding affinity. In fact, although the binding affinity was slightly decreased in the presence of serum (0.1-0.5%), the peptide-displayed phage clones (R3#10) have still binding to DENV2 NS1 protein (Fig. S1 in Supplementary Data).

3.6. Selectivity test

To determine the selectivity of the peptide, similar experiments were performed with M13 wild type phage and BSA. Fig. 4a shows relative binding of R3#10 phage and M13 wild type phage to DENV2 NS1 proteins. A large increase in the impedance was observed upon addition of R3#10 phages, while a small change in the impedance was observed on adding the control, M13 wild type. The small impedance change seen with M13 wild type might be due to non-specific binding of DENV2 NS1 to the peptide. To further confirm selectivity of the R3#10 phage, similar EIS experiments were carried out with BSA and DENV2 NS1 protein. As shown in Fig. 4b, a larger impedance increase in EIS was observed upon addition of DENV2 NS1 (at 25 μg/mL), while a small impedance change was observed upon addition

of BSA as a control (at 34.5 μg/mL). More interestingly, there is no dynamic change of impedance upon addition of dengue virus type 1 (DENV1) NS1 proteins. It was hypothesized that R3#10 phage clones may be no binding affinity since two NS1 proteins used in this study share a 74% sequence similarity. To confirm our prediction, the sequence alignment was performed for each dengue virus NS1, and we observed that nothing relevant was found in both case (Fig. S2 in Supplementary Data). These results indicate that specific and selective binding of DENV2 NS1 protein to the immobilized R3#10 peptide-displayed phage particles.

3.7. Sensitivity test

To determine the sensitivity of the whole phage sensor, the response curve over a range of DENV2 NS1 proteins was measured using EIS. The limit of detection (LOD) obtained by EIS was 1.5 μg/mL. The binding affinity of R3#10 phage clones increased in a concentration-dependent manner. Moreover, R3#10 phage clones showed strong binding affinity to DENV2 NS1 protein at a concentration of as low as 1.5 μg/mL, lower than the normal level of NS1 in bloodstream from dengue patients (50 μg/mL) [2].

3.8. Determination of the dissociation binding constants (K_d) of the multivalent displayed peptides

Fig. 5 shows the equilibrium binding constants of the best peptides for DENV2 NS1 protein. The K_d value of R3#10 was found to be 3.9 ± 0.09 nM, and the results were significantly different. Moreover, the linear range of R3#10 phage was from peptide concentrations of 0.025 to 3.5 μg/mL. Importantly, the newly identified R3#10 peptide-

displayed phage clones showed a limit of detection of 0.025 μg/mL, which is quite comparable or lower than that of other assay systems such as magnetic nanoparticle-based immunoassay (LOD of 25 ng/mL) [31], nanostructured immunoelectrode assay (LOD of 0.035 μg/mL) [32], carbon electrode-based immunosensor (LOD of 0.03 μg/mL) [33] and immunochromatography (sensitivity/specificity was 0.81/0.86) [34]. This is interesting that there are 5 copies of the NS1 recognizing peptide on the surface of whole M13 phage, and this give rise to the dissociation binding constant and lower LOD value. In the point of view in point-of-care applications, peptide-displayed phages are robust and easy to incorporate with other recognition reagents (probes) such as biomolecules, nanoparticles and others. The use of phage particles is fascinating in some aspects; i) mass production of phage is cost-effectively possible, ii) affinity peptides identified by phage display is much smaller in size over antibodies, iii) free peptides away from phage particles can easily be synthesized and created a series of peptide library for the use in biosensor [22,23]. Such our observations, our sensor used in this study may enable the creation of phage sensor as alternative biorecognition element in various biosensing platforms.

4. Conclusions

In summary, unique affinity peptides specific for DENV2 NS1 were successfully identified using M13 phage display technique and characterized using electrochemical detection methods (CV, SWV and EIS). All three detection methods were used for monitoring the binding interactions between peptide-displayed phage clones and DENV2 NS1 protein. Among all the peptides tested, R3#10 (sequence - EHDRMHAYYLTR) showed the greatest decrease in current in CV and maximum increase in impedance in EIS upon binding to

DENV2 NS1 proteins. The R3#10 phage clones were much specific for DENV2 NS1 proteins, as compared with BSA or M13 wild type as control. In EIS, the detection of DENV2 NS1, in accordance with the electron-transfer resistance (R_{ct}) value of the sensor layer, was confirmed, and the R3#10 peptide bound the phage particles with nanomolar K_d values. To our knowledge, this is the first study of discovery and characterization of DENV2 NS1-binding affinity peptides using phage-display technology and electrochemical methods. We showed that the R3#10 phage clones were rich in basic residues (e.g., two His and two Arg), which may be dominantly involved in their binding to DENV2 NS1 protein. We conclude that these results may provide a new bioanalytical approach for detecting and monitoring both dengue virus and DENV fever biomarker. The dengue virus NS1 recognizing peptides are significantly smaller than antibodies, and chemically or genetically manipulated peptides can be easily used for the development of biosensors. However, further validation of this sensor is needed. Firstly, as unmodified peptide-displayed phage particles were used as a potential peptide for detection of a series of dengue virus (type 1-4) NS1 protein, the binding interaction and cross-reactivity should be performed using CV and EIS. Because homologous NS1 or envelope proteins in four dengue serotypes (dengue 1-4) can cause cross-reactions and false-positive outcomes in testing. Therefore, chemical synthesis of synthetic peptides and their characterization in biosensor development using ITC, SPR, and lateral-flow assay could be considered. Secondly, we used DENV2 NS1 protein as target and did not test for real dengue virus because of biosafety issues. Thus, performance of the synthetic peptides with real samples and in the presence of other sample matrixes is currently under investigation.

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Table 1. Sequences of the selected phage clones against dengue virus type 2 NS1 proteins.

Name	**Amino acid (N→C)**	Frequency	Notes
R2 #2	IPLLNPGSMQLS	2/26	Selected after second round
R2 #4	SPPWLPVMPVRA	1/26	Selected after second round
R2 #9	WHYNWQDVSDRQ	3/26	Selected after second round
R2 #10	HSYTQTSLWMKV	1/26	Selected after second round
R3 #10	EHDRMHAYYLTR	2/26	Selected after third round Chosen for characterization

Figure legends

Fig. 1. Selection of peptide-displayed phage clones. To determine relative binding affinities, the selected phage clones were applied onto a gold surface layer and the change in current was measured using square wave voltammetry.

Fig. 2. Selection of peptide-displayed phage clones. Relative binding affinities of the selected phage clones applied onto a gold surface layer were determined from the change in impedance, measured using EIS.

Fig. 3. Effects of different DENV2 NS1 proteins (a, c) and R3#10 phage (b, d) concentrations on the binding interactions. DENV2 NS1 protein concentrations were varied from 1.5 μg/mL to 25 μg/mL, and the change in impedance was monitored using EIS (a, c) and square wave voltammetry (c, d), respectively. A similar experiment was performed with varying concentrations of 3#10 phage clones (10^5 to 10^9 copy/mL), and the change in impedance and current was monitored using EIS and square wave voltammetry.

Fig. 4. Selectivity test of selected phage clones (R3#10). (a) 100 μL of phage particles (10^9 pfu/mL of R3#10 and M13 wild type) were incubated with DENV2 NS1 proteins (25 μg/mL) for 1 h at room temperature. (b) Phage particles (100 μL, 10^9 pfu/mL of R3#10) were incubated with dengue virus type 1 NS1 (DENV1 NS1) and type 2 NS1 (DENV2 NS1) protein (25 μg/mL) or BSA as a control (34.5 μg/mL) for 1 h at room temperature. Changes in impedance in both cases were measured using EIS.

Fig. 5. Equilibrium dissociation constants (K_d) of the selected phage clones. Detailed experimental conditions were shown in the Experimental section. All measurements were done in 5 times with separate electrodes, and error bars represent standard deviations.

Fig. 1

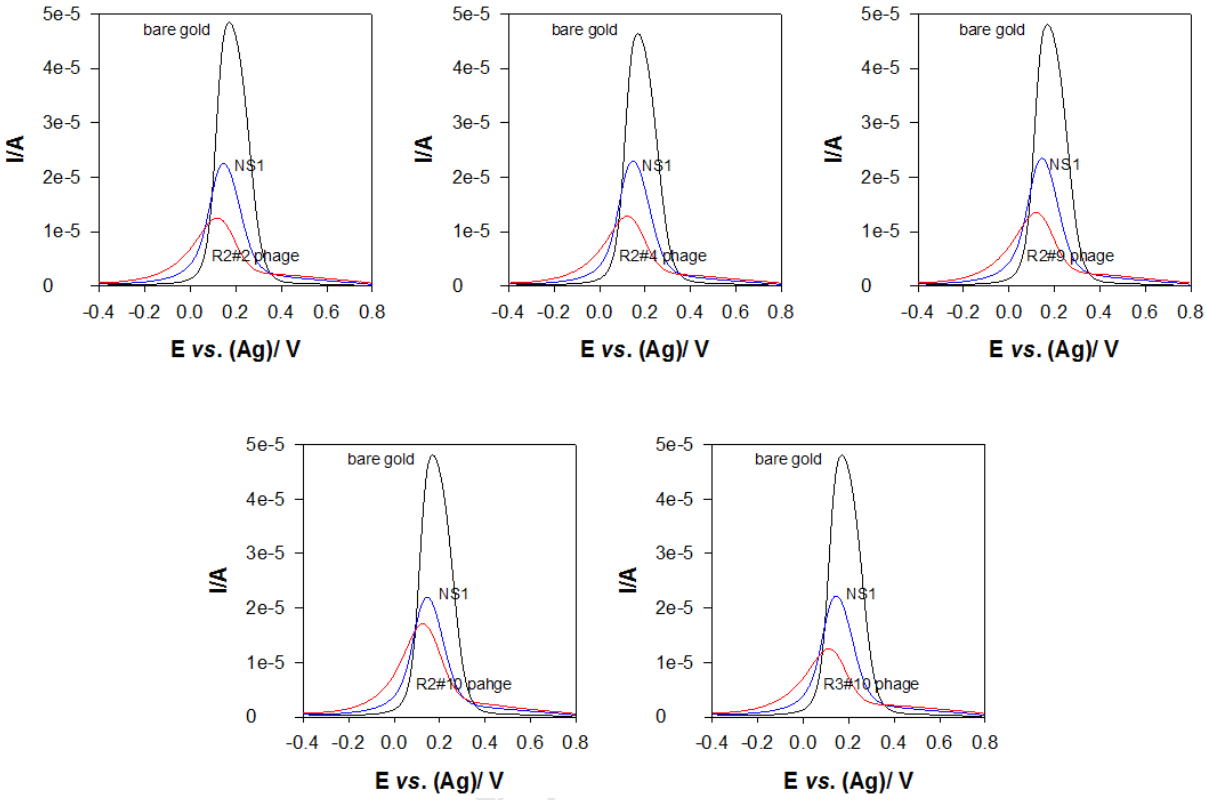


Fig. 2

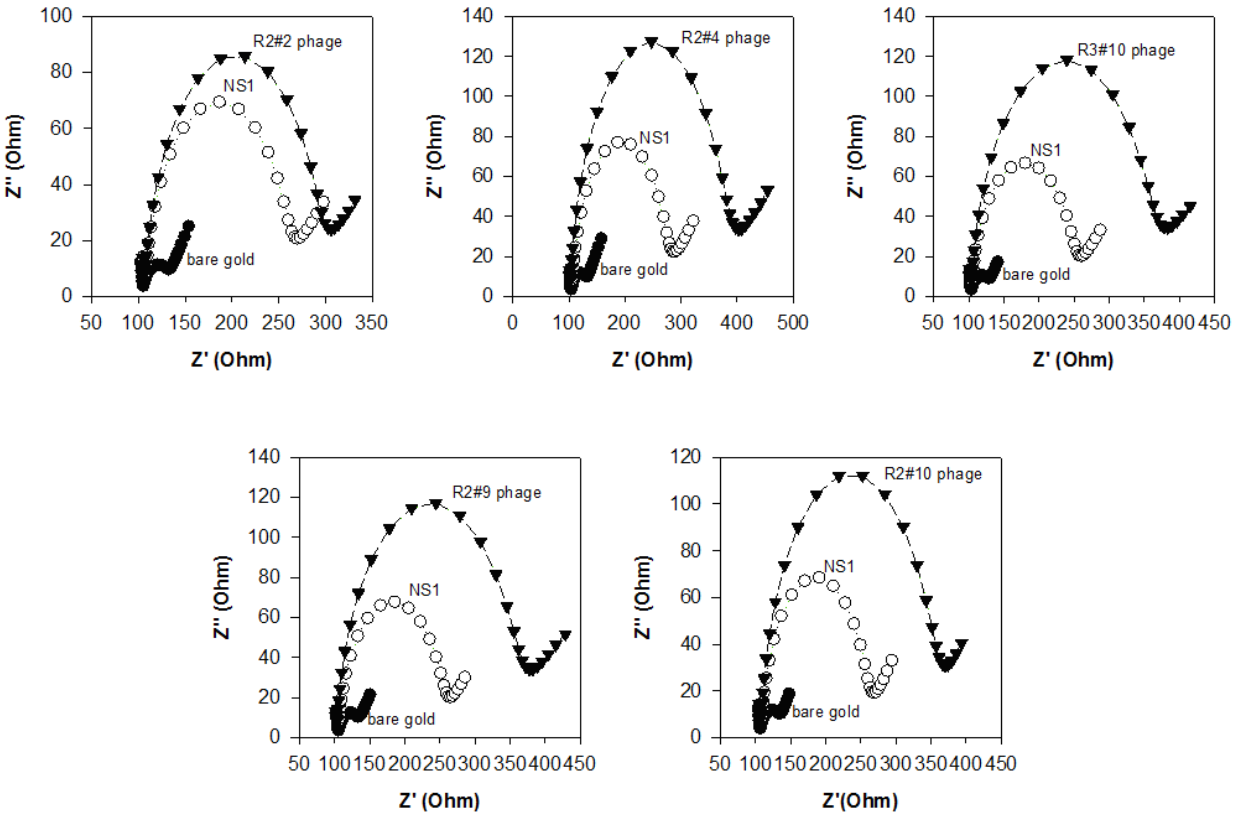


Fig. 3

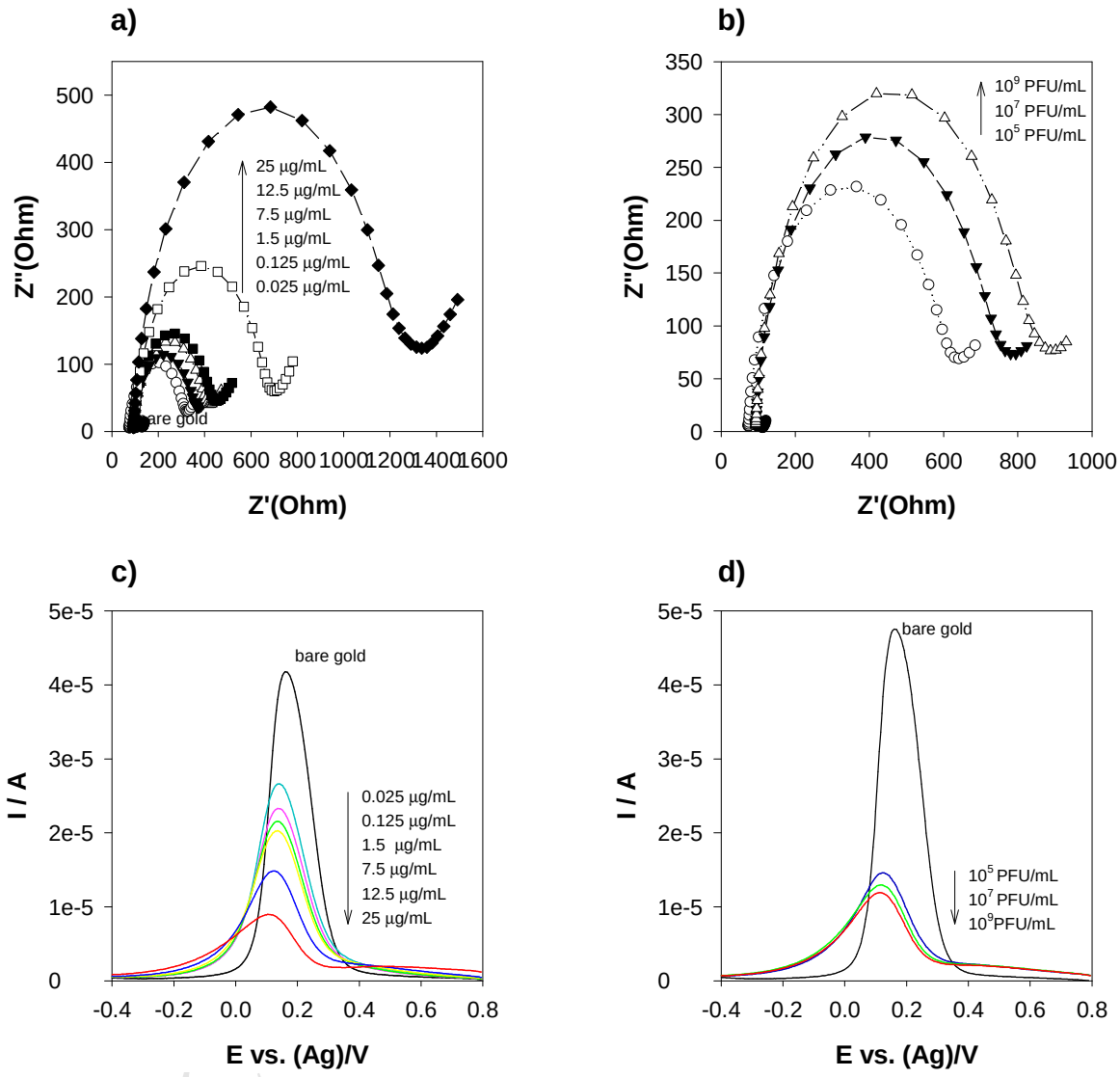

a)
Z''(Ohm)
Z'(Ohm)
25 µg/mL
12.5 µg/mL
7.5 µg/mL
1.5 µg/mL
0.125 µg/mL
0.025 µg/mL
bare gold
b)
Z''(Ohm)
Z'(Ohm)
10^9 PFU/mL
10^7 PFU/mL
10^5 PFU/mL
c)
I / A
E vs. (Ag)/V
bare gold
0.025 µg/mL
0.125 µg/mL
1.5 µg/mL
7.5 µg/mL
12.5 µg/mL
25 µg/mL
d)
I / A
E vs. (Ag)/V
bare gold
10^5 PFU/mL
10^7 PFU/mL
10^9PFU/mL

Fig. 4

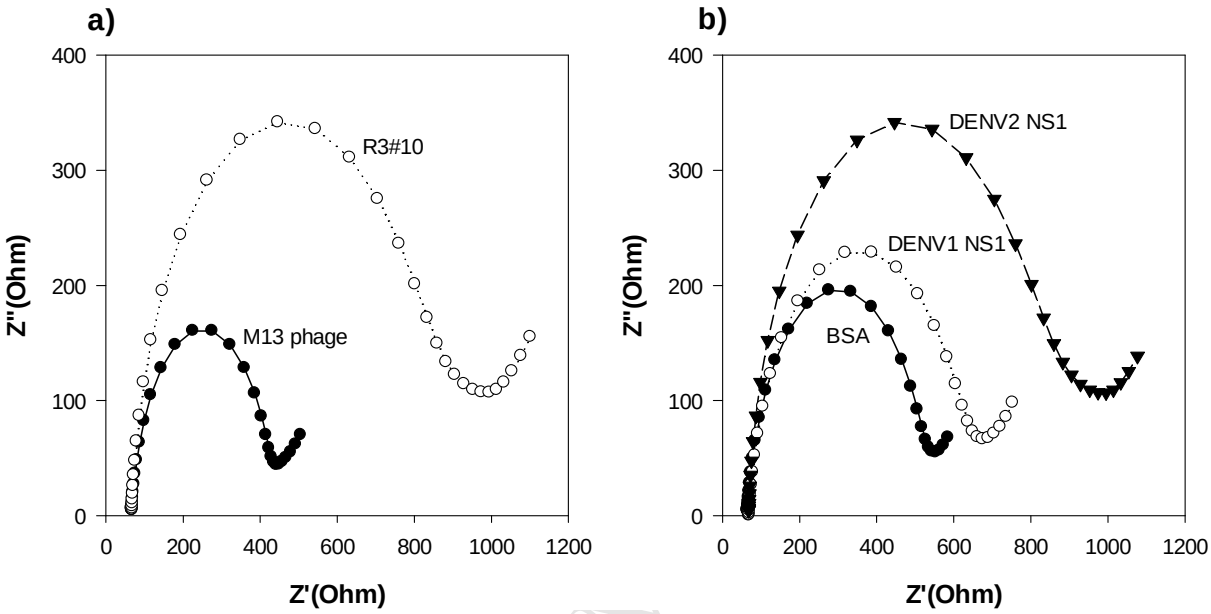
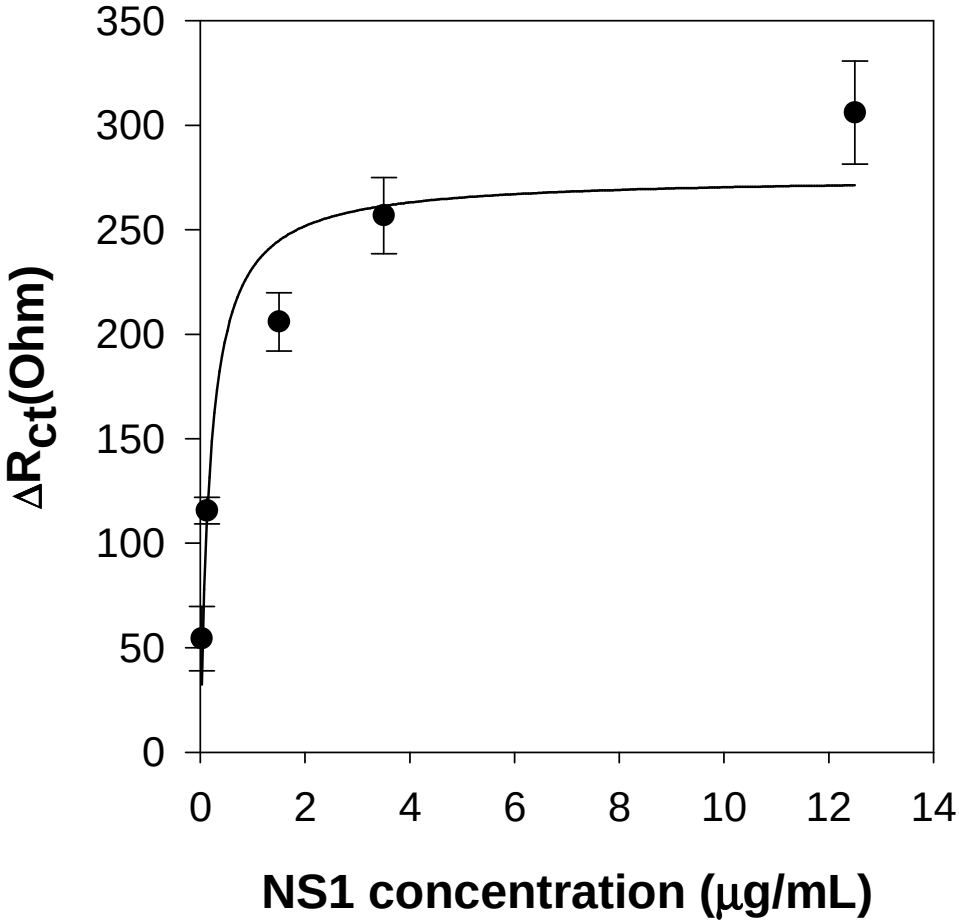


Fig. 5


350
300
250
200
150
100
50
0
ΔRct(Ohm)
0
2
4
6
8
10
12
14
NS1 concentration (μg/mL)

Highlights

- An ultrasensitive and label-free electrochemical biosensor was developed for detection of dengue fever biomarker NS1
- The detection performance of electrochemical biosensor was accessed by CV, SWV and EIS.
- The detection limit with unmodified peptide-displayed phage was found to be 0.025 μg/mL.