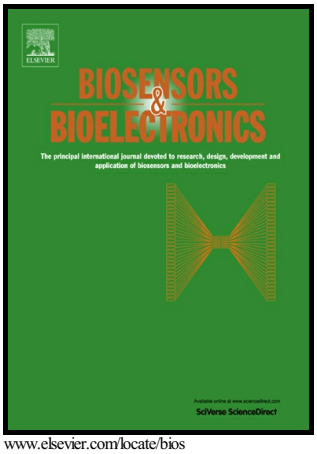

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High sensitive and selective electrochemical biosensor: Label-free detection of human norovirus using affinity peptide as molecular binder

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ABSTRACT

Norovirus is known as the major cause of highly infection for gastrointestinal tracts. In this study, robust and highly sensitive biosensors for detecting human norovirus by employing a recognition affinity peptide-based electrochemical platform were described. A series of amino acid-substituted and cysteine-incorporated recognition peptides isolated from evolutionary phage display technique was chemically synthesized and immobilized to a gold sensor layer, the detection performance of the gold-immobilized synthetic peptide-based sensor system was assessed using QCM, CV and EIS. Using EIS, the limit of detection with Noro-1 as a molecular binder was found to be 99.8 nM for recombinant noroviral capsid proteins (rP2) and 7.8 copies/mL for human norovirus, thereby demonstrating a high degree of sensitivity for their corresponding targets. These results suggest that a biosensor which consists of affinity peptides as a molecular binder and miniaturized microdevices as diagnostic tool could be served as a new type of biosensing platform for point-of-care testing.

***Keywords**:* Human norovirus, affinity peptide, molecular binder, limit of detection, sensitivity, biosensor

1. Introduction

Norovirus is the most common cause of foodborne-disease outbreaks worldwide. Norovirus exposure occurs mainly from contaminated water or polluted food, and infection can rapidly spread from person to person through fecal-oral routes (Beier et al., 2014; Bu et al., 2008; Chung et al., 2015). Infection with norovirus often occurs with relatively low amounts of virus, as low as 1×10^2 copies/mL (Yakes et al., 2013), however, there are no reports of development of vaccines or tremendous anti-noroviral agents. Considering these issues, a platform that yields rapid, sensitive, and accurate detection of norovirus is desirable (Han et al., 2014; Kim et al., 2015). Currently, there are several methods utilized for efficient detection of the relative levels of norovirus in a sample, such as nucleic acid amplification (Ishida et al., 2008), antibody-based immunoassay (Hong et al., 2015; Yoda et al., 2000), real-time PCR (Suffredini et al., 2011), and other methods (Kwon et al., 2010; Yakes et al., 2013). However, these methods require multiple steps, a long time for sample preparation, and relatively expensive analytical instruments (Park et al., 2010). One of the major issues with these methods is that they are not ready-to-use with high sensitivity and are not suitable for point-of-care clinical applications.

Many studies have focused on providing promising alternative methods for more sensitive and accurate detection of norovirus, such as aptamer-based electrochemical biosensing platforms (Beier et al., 2014; Giamberardino et al., 2013) and miniaturized microfluidic biochips (Chung et al., 2015). In fact, the miniaturized electrochemical detection technique has been widely used for the creation of straightforward label-free diagnostic biosensors capable of real-time monitoring (Wu et al., 2010; Wu et al., 2011; Zhang et al., 2014). The advantages of using an electrochemical method are that it is cost-effective,

produces a fast response, is label-free, and is easy to integrate into miniaturized microdevices like portable biosensors (Hong et al., 2015; Shin et al., 2011; Wu et al., 2011; Yea et al., 2016). For example, quartz crystal microbalance (QCM) has been widely used in biosensor development (Geng et al., 2008; Ogi et al., 2011; Wu et al., 2011). With this system, two parameters including frequency (related to mass and thickness) and dissipation (related to rigidity) are measured simultaneously in real time on the sensor surface (Wu et al., 2011). In addition, both cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) are widely used to measure either the current or the impedance obtained by varying the applied potential (Geng et al., 2008; Wu et al., 2011) upon the binding of targets. Therefore, there is no doubt that electrochemical-based bioanalytical techniques are powerful tools for studying molecular binding events such as peptide-protein interactions on a gold surface.

In aforementioned above, current antibody-based immunoassay has been used for clinical applications. This method relies on antibodies as affinity element and is relatively expensive and multi-step processing for sample preparation. Polyclonal antibodies are relatively cheaper, but heterogeous to binding properties, while monoclonal antibodies are expensive to produce, but homogeneous. In modern clinical pratice, more rapid, sensitive and cost-effective diagnoses for norovirus are crucial. Although the progress has been made in ready-to-use applications, improved biosensing platforms are still needed. Several studies have addressed these pitfalls on the affinity element with the use of peptide (Park et al., 2010; Wu et al., 2010), peptide nuleic acid (Mateo-Marti et al., 2007; Wang et al., 2009), aptamer (Kwon et al., 2010) and nanobody (Chen et al., 2016). In comparison to the molecular weight or size of antibodies, peptides are relatively small, and it can be cost-effectively synthesized. The molecular structures of antibodies are standard units with different isotypes, however,

peptides are linear or cylic, indicating that peptides are more amenable over antibodies to engineering at the molecular level (Hwang et al., 2015). As we previously described (Hwang et al., 2015), unique and short linear peptides specific for recominant noroviral capsid proteins (rP2) was identified with evolutionary phage display and characterized by ELISA assay. As we previously described (Hwang et al., 2015), P-domain in noroviral capsid proteins connects with the internal S-domain, and it is exposed on the outermost viral surface. In addition, P-domain is involved in norovirus-host cell binding and possesses immunogenic activity (Bereszczak et al., 2012; Bu et al., 2008), indicating that P2 domain has enough potential to be a good candidate for recognizing and detection of norovirus. Based on the our results, the binding affinity of the peptide-displayed phage particles was found to be a nanomolar range against rP2 proteins. Therefore, we have chosen the best peptides specific for noroviral proteins and further explored the performance of the biosensor for electrochemical detection for human norovirus. In this study, we demonstrate an effective approach by creating a novel peptide-based small-molecule electrochemical biosensor for the detection of human norovirus.

2. Materials and methods

2.1. Chemicals

Fecal bovine serum (FBS) and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The synthetic peptides modified with a C-terminal cysteine and a linker (-GGGGS-) (Noro-1 sequence: QHKMHKPHKNTKGGGGSC, Noro-2 sequence: QHIMHLPHINTLGGGGSC) were synthesized (> 95% purity) by Peptron (Daejeon, Korea).

The quartz crystals deposited with gold was obtained from Biolin Scientific (Stockholm, Sweden). Phosphate-buffered saline (PBS, pH 7.4) and Tris-HCl buffer (pH 7.4) solutions were used to make all protein (1-10 μg/mL) and peptide (0.1 mM) solutions for the QCM, CV and EIS measurements.

2.2. Preparation of virus samples

Human norovirus from Chung-Ang University (Korea) was confirmed in patient stool by real-time reverse transcription polymerase chain reaction. Its genotype was determined as norovirus GII.4 subtype by sequence analysis. The patient stool was mixed with 1:10 ratio of serum-free Dulbecco's modified Eagles medium (DMEM) and homogenized using vertex shaker (Genius 3, IKA, USA) for 5 min. The homogenized sample was centrifuged at 10,000 ×g, 4ºC for 15 min. The supernatant was collected and serially filtered with 0.8 μm, 0.45 μm, and 0.2 μm low-protein binding filter (Millipore Corporation, Billerica, MA, USA). The norovirus titer was determined as 1×10^7 virus particles/mL by quantitative real-time reverse transcriptase-polymerase chain reaction (RT-qPCR). More detailed virus preparations including quality test by RT-qPCR were described in a previous report (Choi and Kingsley 2016; Lee et al., 2015).

Human rotavirus strain Wa from Centers for Disease Control & Prevention (Osong, Korea) was cultured on MA-104 cells from American Type Culture and Collection (ATCC, Manassas, VA, USA) according to our previous report (Seo et al., 2014). After centrifuging the medium at 10,000 ×g, 4ºC for 5 min, and the supernatant was aliquoted and used as a human rotavirus.

2.3. Circular dichroism (CD) spectroscopy

The CD spectra of all of peptides solutions with a concentration of 50 μM were recorded on a circular dichroism spectrometer (J-715, JASCO, Tokyo, Japan) using a UV cell with 0.1-cm of optical path length at 25°C. In the CD experiments, PBS solution (pH 7.4) was used, and the CD spectra were added for 4 times, as previously reported (Greenfield 2006; Sawada et al., 2013).

2.4. Preparation of affinity peptide-modified working electrode

Peptide-functionalized gold working electrode was prepared by the following steps. Firstly, the gold electrode was immersed into piranha solution (H_2SO_4: H_2O_2= 4:1, v/v) for removing dusts and impurities on the surface of the gold electrode for about 10 min. Then, the electrode was rinsed with deionized (DI) water several times and sonicated in the water during 1 min. This pre-treated electrode was dried by blowing N_2 gas for several seconds. After polishing steps, the electrode and voltammetric cell were assembled together. 100 μL of thiol-modified peptide (50 μg/mL) was dropped onto the gold electrode and incubated in room temperature for overnight. In order to remove unbound peptides, this cell was washed with 1X PBS (pH 7.4) and then DI water. The peptide-bound electrode was coated using a Pt using a sputter-coater (COXEM, KIC-1A, Seoul, Korea) to avoid charge-up effect of peptides on the surface. The surfaces of gold electrodes were observed using field-emission scanning electron microscopy (FE-SEM, Hitachi S-4800, Tokyo, Japan). Next, 30 μL of serially-diluted norovirus samples (10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 copies/mL) were loaded on the

each assembled cell and then incubated at room temperature for 3 h. In order to remove the unbounded virus, these cells were sequentially washed with 1X PBS and DI water.

2.5. QCM measurements for determination of binding interactions on gold-immobilized peptide sensor layer

The QCM measurements were conducted using a Q-Sense E1 instrument (Biolin Scientific, Stockholm, Sweden). This instrument consists of a very thin quartz disc with gold electrode on both sides. The QCM measurements were conducted at room temperature, and a stable baseline was achieved 1 h after flow injection on a clean crystal at a flow rate of 1 mL/min. Briefly, the gold electrodes were prepared in 0.1 mM peptide solution for 1 h to obtain a gold-Noro-1-Cys or gold-Noro-2-Cys electrode. After washing two steps, the Au-peptides electrodes were incubated in target protein solution for 1 h before QCM measurements. The Voigt viscoelastic model was used to calculate the thickness of peptide immobilized on the sensor surface in terms of both frequency and dissipation, as previously reported (Liu et al., 2003; Ogi et al., 2011; Sawada et al., 2013).

2.6. CV and EIS measurements for detection of human norovirus

A conventional three-electrode cell including the above-mentioned gold working electrode, platinum counter electrode, and Ag/AgCl reference electrode was used for the electrochemical measurement. CV and EIS were performed using an electrochemical analyzer (CHI 750E, CH Instruments, Austin, TX, USA), connected to a computer data analysis system. These analyses were conducted in a PBS solution with 4 mM of ferro/ferricyanide. The CV was recorded from 0.0 V to +0.4 V vs. Ag/AgCl electrode at

sweep rate of 20 mV/s. 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.

3. Results and discussion

3.1. The detection performance of affinity peptide-based electrochemical biosensor

To begin to evaluate the affinity peptide-based sensor system, a series of cysteine-incorporated free peptides specific for rP2s identified from phage particles was chemically synthesized and immobilized onto the gold surface, the binding events were monitored in situ by QCM, CV, and EIS (Scheme 1). In detail, the Noro-1 peptide has an amino acid sequence of QHKMHKPHKNTKGGGGSC, and we incorporated a cysteine residue (Cys) at the C-terminus for a thiol self-assembled monolayer and a linker (-GGGGS-) for molecular flexibility on the gold surface. In order to determine the effects of amino acid composition on binding interactions, Lys residues of the Noro-1 were replaced by neutral and nonpolar amino acids (Ile and Leu) to create the Noro-2 peptide. They have an amino acid sequence of QHIMHLPHINTLGGGGSC. Briefly, the gold electrodes were prepared in 0.1 mM of each peptide solution for 1 h, followed by backfilling in 1 mM cysteine solution for 2 h to obtain gold-Noro-1-Cys or gold-Noro-2-Cys electrodes. The functionalized gold electrodes were incubated with rP2 proteins for 30 min at room temperature before QCM and EIS measurements. Fig. 1 shows the specific immobilization of the synthetic peptides and their binding properties on the gold surface. As expected, Noro-1 peptides were specifically immobilized on the QCM substrate, as observed by a drop in frequency change and an increase in dissipation change when the Noro-1 peptide was added. Relative saturation was reached around 1 h upon addition of the Noro-1 peptide solution. The Noro-1-Cys was immobilized to gold particles via gold-thiol bond formation, which formed the self-assembly

monolayer (SAM). The immobilized Noro-1-Cys peptide was monitored using FE-SEM analysis (Fig. S1). The bare gold electrode exhibites an original gold-sputted surface morphology, compared with the peptide-modified gold substrate. SEM investigations reveal the difference of the surface morphology between the gold and peptide-coated gold samples. After Noro-1 peptide binding, partial aggregates of the peptide were found on the gold surface, which confirmed the self-assembled peptide layer (Fig. S1b). This phenomenon is consistant with previous study (Wu et al., 2010) and well-documented. This is the fact that the SAM tethered with carboxylic acid group is attributed to be functionalized for peptide or antibody immobilization by Au-S bond (Lee et al., 2007).

In QCM experiments, the quick frequency drop due to peptide immobilization was about 140–160 Hz, which corresponds to an areal mass of 4068 ng/cm^2 based on a peptide molecular weight of 1932 g/mol (Fig. 1a, 1b). Surprisingly, the dissipation change also quickly increased by 10–12 × 10^{-6} after addition of rP2 proteins. According to the Voigt viscoelastic calculation model (Sawada et al., 2013), the film thickness was found to be ~27 nm in height. Upon binding of rP2 proteins by the immobilized peptides, the subsequent washing after introduction of Noro-1 peptide and target proteins caused a small frequency increase. This could be due to several reasons, including the detachment of loosely bound peptide or the change in physicochemical properties such as viscosity, density or peptide mobility in whole solutions (Wu et al., 2011). Fig. 1c and d show the immobilization of Noro-2 peptides and the performance of biosensor on the sensor surface. Introduction of the Noro-2 peptide resulted in a frequency change in the QCM signal. However, relative saturation was not reached when Noro-2 peptide was added. More interestingly, since Noro-2 peptide has Ile and Leu amino acids that Noro-1 does not, the film thickness and areal mass were found to be

~22.7 nm and 3409 ng/cm^2, respectively. These values are less than those obtained from Noro-1, indicating that as a result of the substituted peptides, Noro-2 showed much lower affinity for target proteins than Noro-1. We hypothesize some possible reasons for this; first, the replacement with two neutral amino acid residues, Ile and Leu, may decrease the overall positive charge and reduce binding affinity for target proteins. Second, the substitution of Lys at positions 3, 6, 9, and 12 may cause a conformational change in the peptides. In fact, the relative binding ability was decreased after substitution of four Lys residues, possibly due to the disappearance of positively charged residues in the peptides, suggesting that positively charged Lys residues are essential for target recognition and binding.

3.2. Effects of serum as interferent on binding interaction

To verify the specificity of synthetic peptides for binding of the targets, 1% (v/v) fetal bovine serum was spiked into pre-immobilized peptides with rP2 proteins on the gold surface and QCM signal was measured (Fig. 2). In case of Noro-1, the frequency quickly decreased by 100–120 Hz, while the dissipation change increased 9–12 × 10^{-6}. In spiked samples with serum, the peptide binding affinities were not significantly affected; however, they still exhibited binding to rP2 proteins (Fig. 2a, 2b). In the presence of 1% serum, the film thickness and areal mass of Noro-1 were found to be ~19 nm and 2730 ng/cm^2, respectively. These results demonstrate that affinity peptide, Noro-1 has high selectivity for rP2 proteins. Unfortunately, in the case of the Noro-2 peptide, the binding affinity during subsequent immobilization of peptides with serum and washing with buffer was significantly decreased (Fig. 2c, 2d). In fact, no significant change in frequency or dissipation was observed. This may be due to bioactive compounds or residual ingredients in serum acting as inhibitors of

binding interactions, resulting in non-specific binding with the peptides.

3.3. Effects of structure-to-function of amino acids in affinity peptides on binding interaction

To study the effects of peptide secondary structure on binding events, the two peptides were analyzed by CD spectroscopy. The spectrum between Noro-1 and Noro-2 peptides was similar but not identical (Fig. S2). These two peptides can both form a random coil structure. It is worthy to note that while the Noro-2 peptide was able to form a random coil structure, its binding affinity was dramatically decreased, suggesting that both positively charged Lys residues and a random coil structure are necessary for specific binding to norovirus surface proteins. In addition, Noro-1 contains a Pro residue at position of 7 which may introduce a sructural kink that is essential for efficient binidng of the target proteins (Wu et al., 2011).

3.4. Evaluation of sensitivity and selectivity of biosensor system

To study the feasibility of the recognition peptides, similar experiments were performed with EIS. The dynamic response curves over a range of rP2 protein concentrations (0.01–1000 μg/mL) are shown in Fig. 3 and Fig. S3. All the experiments were repeated three times with similar results each time. Upon binding of target proteins by the gold-immobilized peptides, a large increase in impedance due to surface blocking was observed in a concentration-dependent manner. A similar result was observed in our previous studies (Wu et al., 2011). The limit of detection (LOD) of Noro-1 peptide was 1.44 μg/mL (99.8 nM), while the Noro-2 peptide had a LOD of 3.94 μg/mL (273.6 nM) by the 3σ-rule, indicating that Noro-1 peptides had a much higher affinity than Noro-2 peptides.

To detect norovirus obtained from patients with diarrhea, the electric current change with

various norovirus concentrations (10^1 to 10^7 copies/mL) was measured as shown in Fig. 4. CV and EIS measurements validated the formation of the peptide- based sensing layer. As expected, when norovirus was specifically bound to the immobilized Noro-1 peptide, the currents decreased in response to the elevated norovirus concentration, while no distinct changes in the electric current appeared with increasing rotavirus concentrations (Fig. 4c and 4f, and Fig. S4 and S5). Upon binding of norovirus by the immobilized peptide, a large impedance increase was observed with EIS (Fig. 4d and Fig. S6). No change was observed for the rotavirus negative control in the same conditions (Fig. 4e and Fig. S7). The data suggests that the Noro-1 peptide has specific binding capability for GII.4 norovirus. Based on the EIS response curve, the LOD was found to be 7.8 copies/mL by the two-segment linear relationship (3σ/slope), according to the previous report (Cui et al., 2016). EIS experiments were repeated three times with similar results each time, and these results were quite reproducible. Comparing these results to those described in the literature (Table S1), the new biosensor had a LOD value just below the infectious dose of norovirus (i.e., $<10^2$ viral particles), and this LOD value was fond to be superior lower level than that obtained using continuous-flow reverse transcription-PCR (6.4×10^7, Li et al., 2011), IDEIA norovirus kit (1.6×10^7, Costantini et al., 2010), and rapid immunochromatographic kit (3.2×10^6, Pombubpa and Kittigul, 2012).

To test the selectivity of the norovirus sensor, norovirus and rotavirus with respective concentrations of 1×10^4 copies/mL and 1×10^4 pfu/mL were separately spiked onto the sensing layer and electrical impedance change was measured. The impedance change only appeared with norovirus samples, while the difference between peptide alone and spiked rotavirus was almost equal (Fig. 4f and Fig. S8 and S9). Therefore, the data suggest that the

newly identified Noro-1 peptide is strongly specific and selective to GII.4 norovirus. Another benefit to this approach is that linear, short recognition peptides identified using evolutionary phage display coupled with electrochemical methods can be easily applied for biosensor development. As an example of this approach, we have recenlty demonstrated a new affinity peptide sensor for the detection of procalcitonin and colorectal cancer biomarker (Hwang et al., 2016, Park et al., 2015). Compared to conventional recognition elements such as antibodies, unique peptides are relatively small and can be cost-effectively synthesized for manipulation at a molecular scale, therefore they are useful for creating new bioanalytical platforms. Therefore, short and unique affinity peptides are being utilized in many arena; the creation of nanostructures (nanofiber, nanotube, and hydrogel) for biomedical applications and the developement of biosensors for clinical applications (Park et al., 2010). More importantly, among all three techniques used in this study, EIS appears to be best suited for electrochemical detection of rP2 protein and human norovirus. Although one of the limitations of EIS is the need to measure the impedance signal in the presence of a redox mediator, EIS technique as monitoring tool is quite straightforward and produced a more linear and quantitative detection range than can be obtained with QCM or CV.

4. Conclusions

In conclusion, we demonstrate the binding affinity and the feasibility of immobilized synthetic peptides on a gold surface as analyzed by QCM, CV, and EIS. In fact, the LOD of the Noro-1 peptide was found to be 99.8 nM against rP2 protein and 7.8 copies/mL against real norovirus from diarrheal patients, which is the most sensitive assay developed so far to our knowledge. Both evolutionary phage display and electrochemical techniques are mature

technologies, and this platform could be used to formulate new biosensors for almost any desired target. All three analytical results demonstrated a quantitative response to a wide range of norovirus concentrations, and thus can be used as a label-free, cost-effective bioanalytical sensing platform for the detection of norovirus from contaminated food or water. Therefore, the results descibed here suggest that the use of affinity peptide as a molecular binder as a new diagnostic element over antibodies is possible. Based on peptide features and its feasibility, the detection of norovirus in shellfish such as oyster or mussel is in progress to evaluate the diagnostic versatility and accuracy of the whole sensor system.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2016.xx.xxx.

References

Beier, R., Pahlke, C., Quenzel, P., Henseleit, A., Boschke, E., Cuniberti, G., Labudde, D., 2014. FEMS Microbiol. Lett., 351, 162-169.

Bereszczak, J.Z., Barbu, I.M., Tan, M., Xia, M., Jiang, X., van Duijn, E., Heck, A.J.R., 2012. J. Struct. Biol., 177, 273-282.

Bu, W., Mamedova, A., Tan, M., Xia, M., Jiang, X., Hegde, R.S., 2008. J. Virol., 82(11), 5340-5347.

Chen, J., He, Q.-h., Xu, Y., Fu, J.-h., Li, Y.-p., Tu, Z., Wang, D., Shu, M., Qiu, Y.-l., Yang, H.-W., Liu, Y.-Y., 2016. Talanta, 147, 523-530.

Choi, C., Kingsley, D.H., 2016. Food Environ. Virol., 8, 141-147.

Chung, S.H., Baek, C., Cong, V.T., Min, J., 2015. Biosens. Bioelectron., 67, 625-633.

Costantini, V., Grenz, L., Fritzinger, A., Lewis, D., Biggs, C., Hale, A., Vinje, J., 2010. J. Clin. Microbiol., 48, 2770-2778

Cui, J., Hu, K., Sun, J.-J., Qu, L.-L., Li, D.-W., 2016. Biosens. Bioelectron., 85, 324-330.

Geng, R., Zhao, G., Liu, M., Li, M., 2008. Biomaterials, 29, 2794-2801.

Giamberardino, A., Labib, M., Hassan, E.M., Tetro, J.A., Springthorpe, S., Sattar, S.A., Berezovski, M.V., DeRosa, M.C., 2013. PLoS One, 8, e79087.

Greenfield, N.J., 2006. Nat. Protoc., 1, 2876-2890.

Han, L., Tan, M., Xia, M., Kitova, E.N., Jiang, X., Klassen, J.S., 2014. J. Am. Chem. Soc., 136, 12631-12637.

Hong, S.A., Kwon, J., Kim, D., Yang, S., 2015. Biosens. Bioelectron., 64, 338-344.

Hwang, H.J., Ryu, M.Y., Park, J.P., 2015. RSC Adv., 5, 55300-55302.

Hwang, H.J., Ryu, M.Y., Lee, G.B., Park, J.P., 2016, ChemistrySelect, 6, 1140-1143.

Ishida, S., Yoshizumi, S., Ikeda, T., Miyoshi, M., Okano, M., Okui, T., 2008. J. Med. Virol.,

80, 913-920.

Kim, Y., Galasiti Kankanamalage, A.C., Chang, K.-O., Groutas, W.C., 2015. J. Med. Chem., 58, 9438-9450.

Kwon, O.S., Park, S.J., Jang, J., 2010. Biomaterials, 31, 4740-4747.

Lee, M., Seo, D.J., Seo, J., Oh, H., Jeon, S.B., Ha, S.-D., Myoung, J., Choi, I.-S., Choi, C., 2015. J. Virol. Methods, 221, 57-61.

Lee, S.W., Chang, W.J., Bashir, R., Koo, Y.M., 2007, Biotechnol. Bioprocess Eng., 12, 185-199.

Li, Y., Zhang, C., Xing, D., 2011. Microfluid. Nanofluid., 10, 367-380.

Liu, Y., Yu, X., Zhao, R., Shangguan, D.H., Bo, Z., Liu, G., 2003. Biosens. Bioelectron., 18, 1419-1427.

Mateo-Marti, E., Briones, C., Pradier, C.M., Martin-Gago, J.A., 2007. Biosens. Bioelectron., 22, 1926-1932.

Ogi, H., Fukunishi, Y., Yanagida, T., Yagi, H., Goto, Y., Fukushima, M., Uesugi, K., Hirao, M., 2011. Anal. Chem., 83, 4982-4988.

Park, J.P., Cropek, D.M., Banta, S., 2010. Biotechnol. Bioeng., 105, 678-686.

Park, J.P., Park, C.Y., Park, A.Y., Ryu, M.Y., 2015, RSC Adv., 5, 90531-90533.

Pombubpa, K., Kittigul, L., 2012. Eur. J. Clin. Microbiol. Infect. Dis., 31, 2379-2383.

Sawada, T., Okeya, Y., Hashizume, M., Serizawa, T., 2013. Chem. Commun., 49, 5088-5090.

Seo, D.J., Lee, M.H., Son, N.R., Seo, S.W., Lee, K.B., Wang, X., Choi, C.S., Food Control, 41, 178-184.

Shin, S., Won, B.Y., Jung, C., Shin, S.C., Cho, D.Y., Lee, S.S., Park, H.G., 2011. Chem. Commun., 47, 6611-6613.

Suffredini, E., Pepe, T., Ventrone, I., Croci, L., 2011. New Microbiol., 34, 9-16.

Wang, Y., Chen, M., Zhang, L., Ding, Y., Luo, Y., Xu, Q., Shi, J., Cao, L., Fu, W., 2009. Biosens. Bioelectron., 24, 3455-3460.

Wu, J., Cropek, D.M., West, A.C., Banta, S., 2010. Anal. Chem., 82, 8235-8243.

Wu, J., Park, J.P., Dooley, K., Cropek, D.M., West, A.C., Banta, S., 2011. PLoS One, 6, e24948.

Wu, J., Mari-Buye N., Muinos T.F., Borros S., Favia, P., Semino, C.E., 2010, J. Nanobiotech., 8, 1-15.

Yakes, B.J., Papafragkou, E., Conrad, S.M., Neill, J.D., Ridpath, J.F., Burkhardt, W. 3rd, Kulka, M., Degrasse, S.L., 2013. Int. J. Food Microbiol., 162, 152-158.

Yea, C.-H., Jeong, H.-C., Moon, S.-H., Lee, M.-O., Kim, K.-J., Choi, J.-W., Cha, H.-J., 2016. Biomaterials, 75, 250-259.

Yoda, T., Terano, Y., Shimada, A., Suzuki, Y., Yamazaki, K., Sakon, N., Oishi, I., Utagawa, E.T., Okuno, Y., Shibata, T., 2000. J. Med. Virol., 60, 475-481.

Zhang, B., Morales, A.W., Peterson, R., Tang, L., Ye, J.Y., 2014. Biosens. Bioelectron., 58, 107-113.

Figure legends

Scheme 1. Work flow diagram for biosensor development. 1, Selection of affinity peptide as molecular binders using phage display technique. 2, Synthesis of synthetic peptide and immobilization. 3, Norovirus addition. 4, Electrochemical detection.

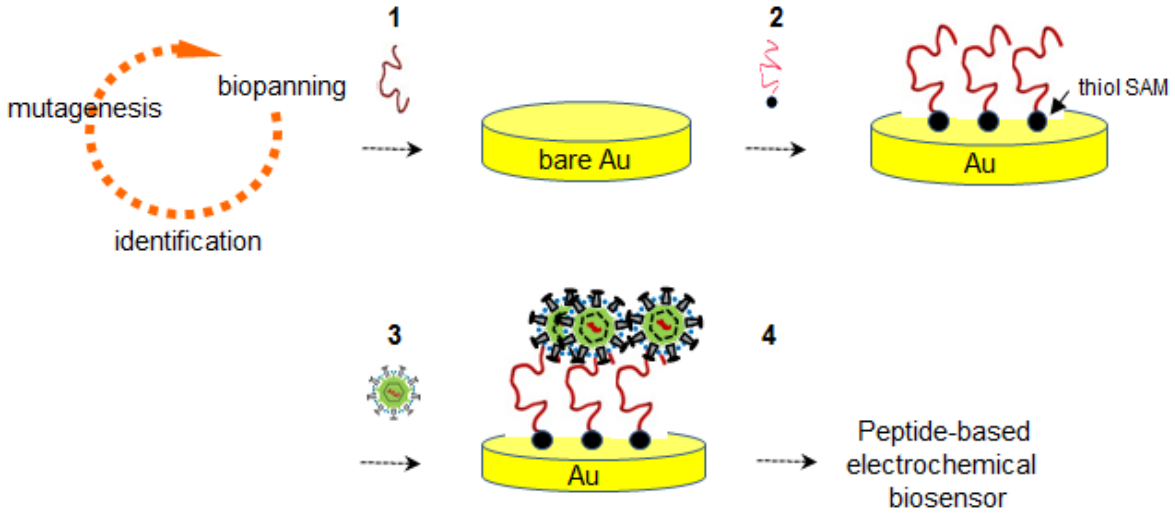


Fig. 1. QCM analysis of binding affinity of the synthetic peptides for rP2 proteins. (a) and (b): Noro-1 peptide, (c) and (d): Noro-2 peptide, I: peptide immobilization, II: PBS washing, III: Tris-HCl buffer washing, IV: introduction of target proteins, V: washing.

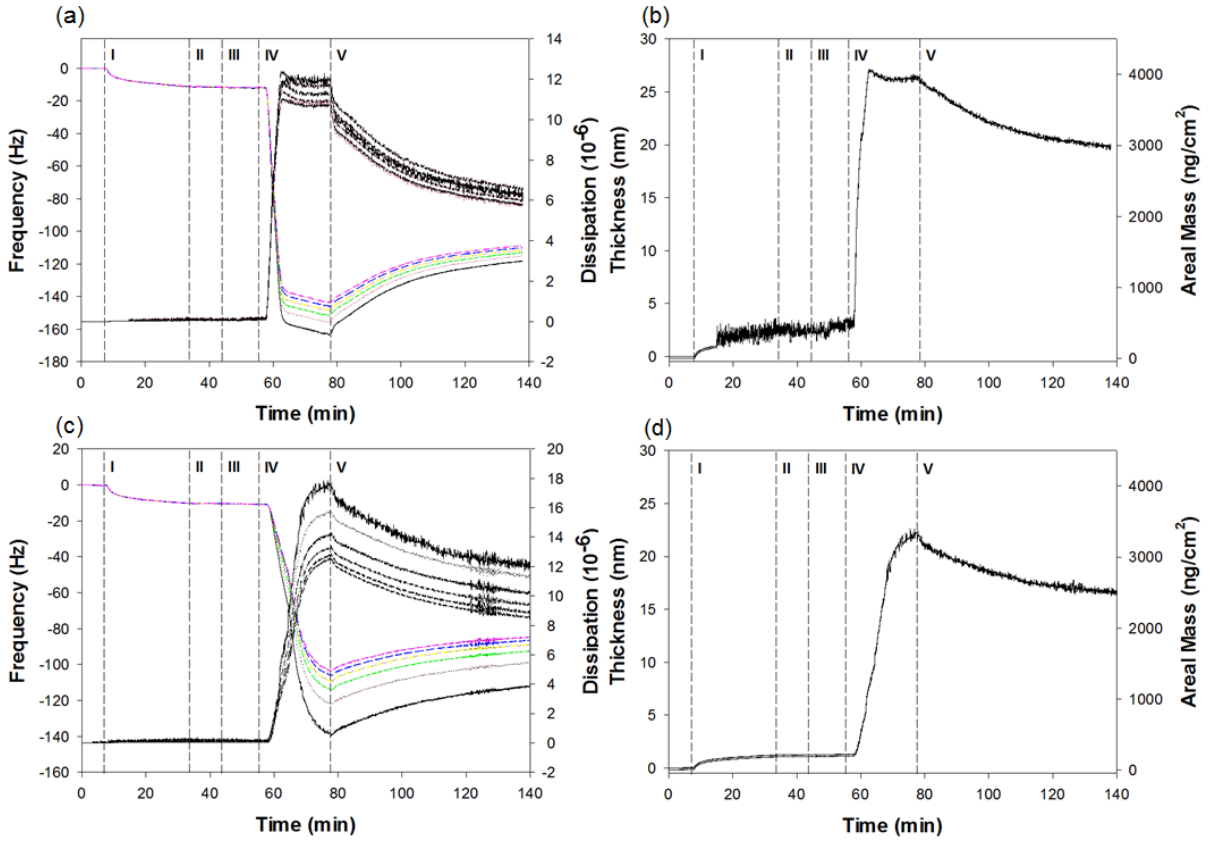


Fig. 2. Effects of serum on binding affinity of the synthetic peptides. (a) and (b): Noro-1 peptide, (c) and (d): Noro-2 peptide. I: peptide immobilization, II: PBS washing, III: Tris-HCl buffer washing, IV: introduction of target proteins with serum, V: washing.

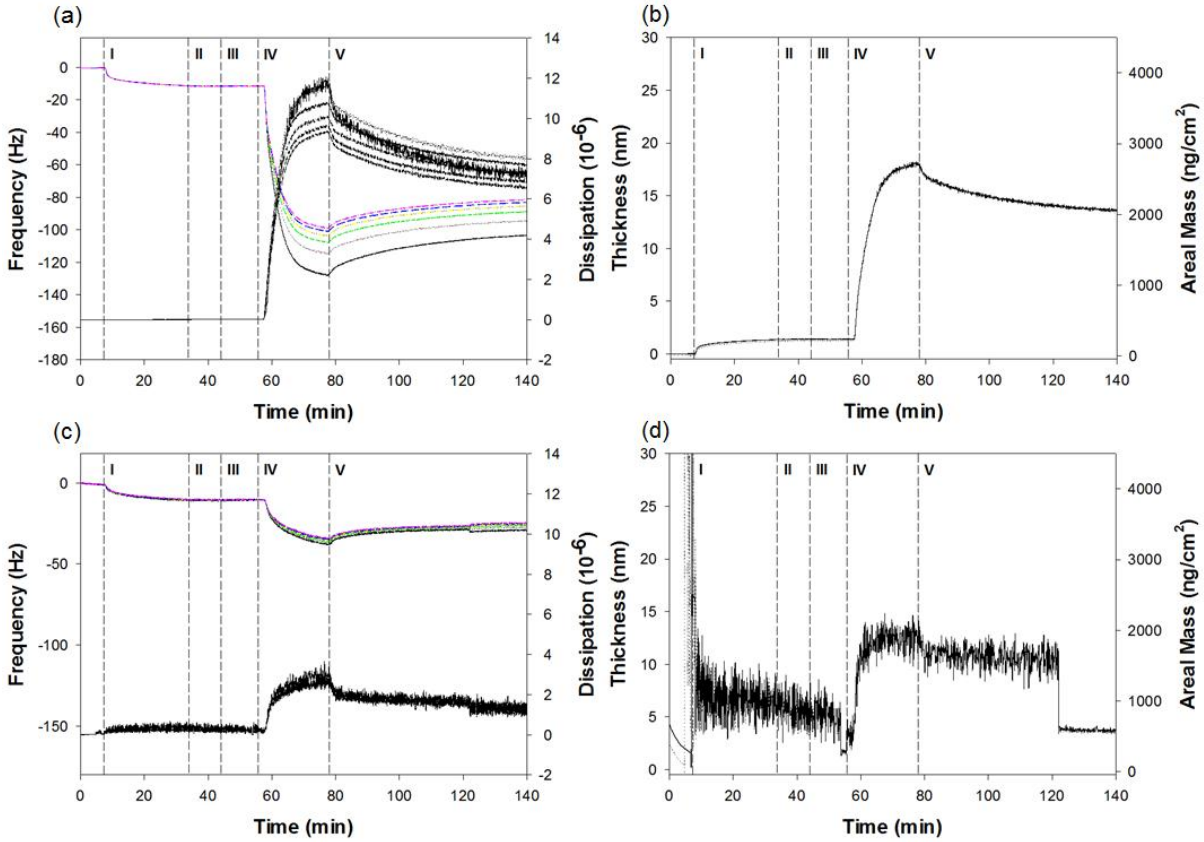


Fig. 3. Dynamic response curves of rP2 proteins binding on gold-immobilized peptides (a; Noro-1 peptide, b; Noro-2 peptide). EIS measurements were performed using a single electrode in protein solutions ranging from low to high concentrations (●; bare gold with peptide, ○; 0.01 μg/mL,▲; 0.1 μg/mL,△; 1.0 μg/mL,■; 10 μg/mL, □; 100 μg/mL,▼; 1000 μg/mL).

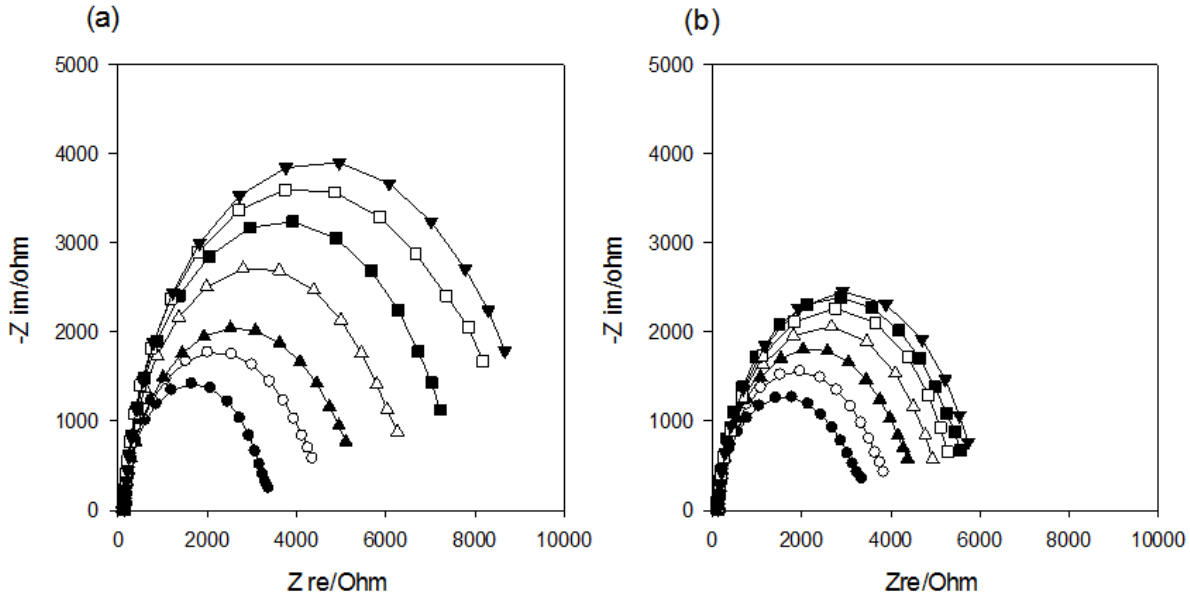
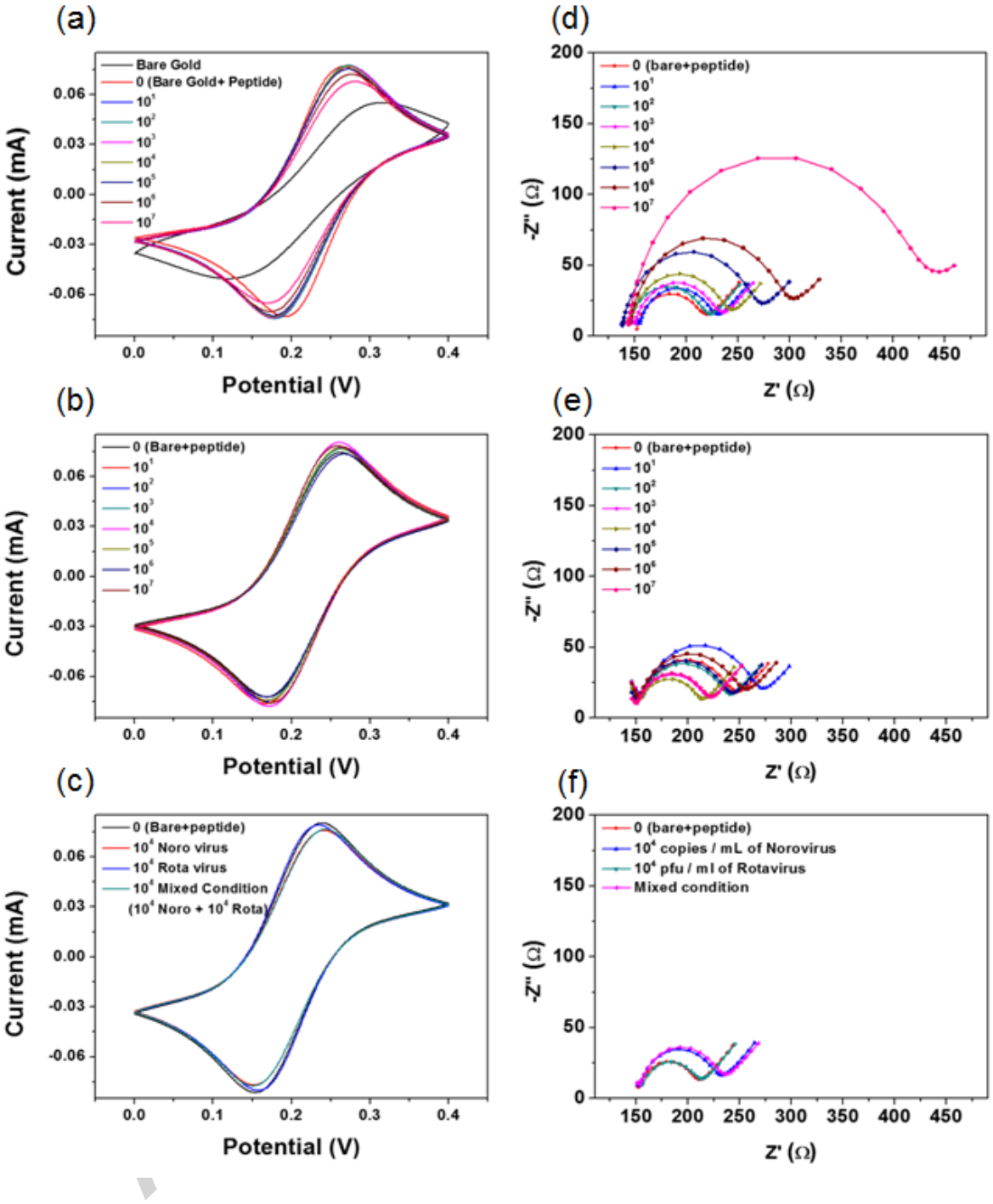
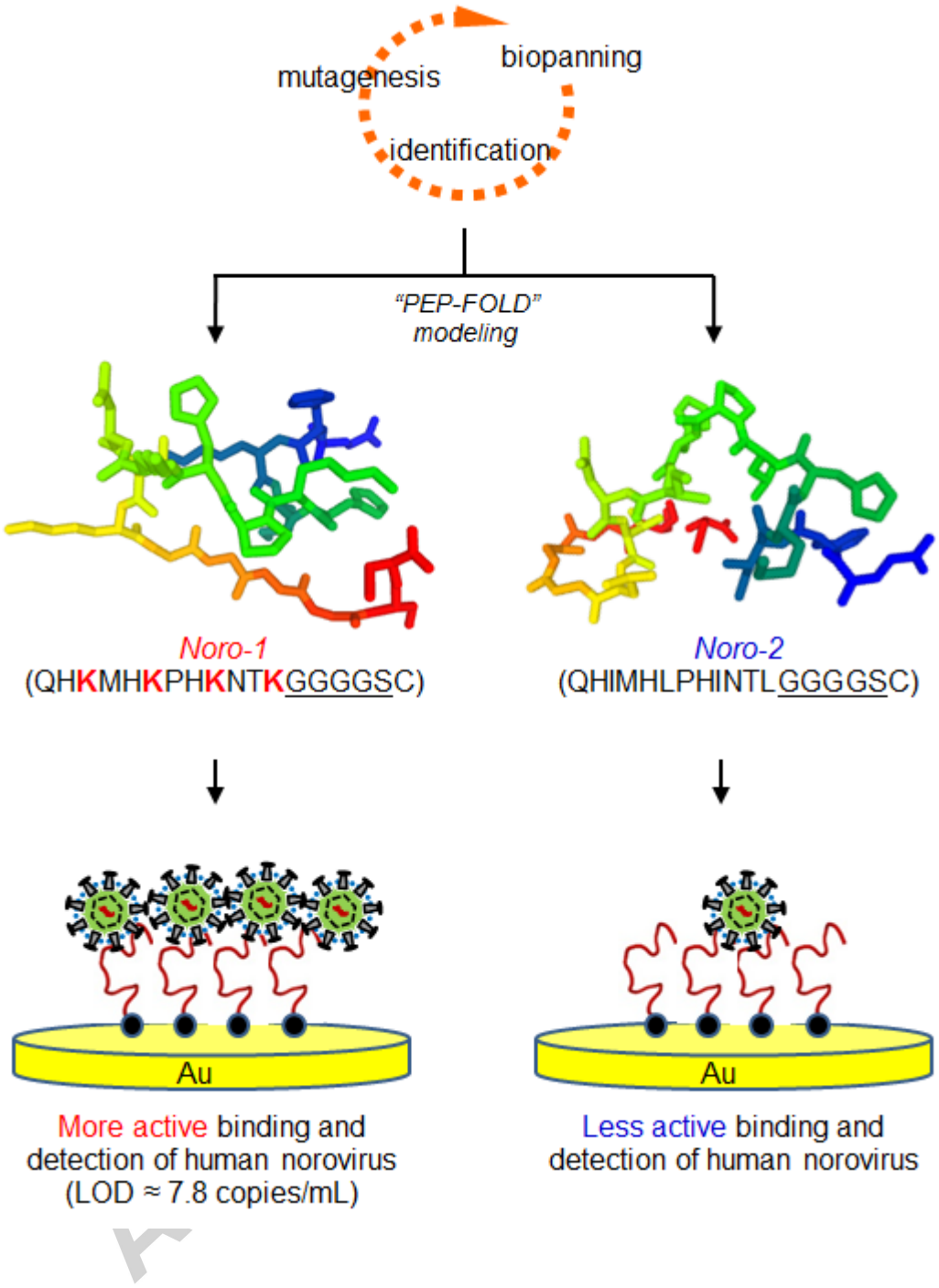


Fig. 4. Electrochemical biosensor operation. (a-c) CV of Noro-1 peptide electrode before and after norovirus binding, (d-e) EIS of Noro-1 peptide electrode before and after norovirus binding. (f) EIS of Noro-1 peptide electrode in mixed norovirus with rotavirus. After incubation with noroviral samples, the electrodes were transferred to 4.0 mM $Fe(CN)_6^{3-/4-}$ in PBS solution for CV and EIS measurements.


(a)
Bare Gold
0 (Bare Gold+ Peptide)
Current (mA)
Potential (V)
(b)
0 (Bare+peptide)
Current (mA)
Potential (V)
(c)
0 (Bare+peptide)
10⁴ Noro virus
10⁴ Rota virus
10⁴ Mixed Condition
(10⁴ Noro + 10⁴ Rota)
Current (mA)
Potential (V)
(d)
0 (bare+peptide)
-Z'' (Ω)
Z' (Ω)
(e)
0 (bare+peptide)
-Z'' (Ω)
Z' (Ω)
(f)
0 (bare+peptide)
10⁴ copies / mL of Norovirus
10⁴ pfu / ml of Rotavirus
Mixed condition
-Z'' (Ω)
Z' (Ω)

GA



Highlights

- An ultrasensitive and label-free electrochemical biosensor for human norovirus was

developed

- The electrochemical detection was performance by CV, QCM and EIS
- The detection limit was found to be 7.8 copies/mL.