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RESEARCH PAPER

Affinity Peptide-guided Plasmonic Biosensor for Detection of Noroviral Protein and Human Norovirus

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Abstract In this study, we developed an affinity peptide-guided plasmonic biosensor that is capable of detection for noroviral capsid proteins and human norovirus. Construction of plasmonic biosensor was achieved by immobilization of affinity peptides (named norovirus binding peptides) on the localized surface plasmonic sensor (LSPR) layer for detection of noroviral capsid proteins and human norovirus. The performance of the plasmonic biosensor in detection of their targets was monitored using LSPR techniques. This specific interaction is proportional to the absorbance of LSPR signals. The lowest detection value for noroviral capsid protein was 0.1 ng/mL in the presence of complex tissue culture media (MEM and FBS), and limit of detection (LOD) for human norovirus was found to be 9.9 copies/mL by the 3-σ rule. Interestingly, no dynamic binding response with norovirus binding peptides as affinity reagent was observed against rotavirus, suggesting that norovirus binding peptides have high selectivity for human norovirus. Thus, norovirus binding peptide-guided plasmonic biosensor could be used for the detection of norovirus-related foodborne pathogens.

Keywords: norovirus, affinity peptide, sensitivity, plasmonic biosensor, limit of detection

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1. Introduction

In point-of-care testing, new biosensing platform techniques are in high demand because of rapid growth of diagnostics, such as disease biomarkers, pathogenic bacteria, and high risk of viruses. Therefore, it is expected to play an important role in clinical or biomedical applications. Among various biosensing methods, localized surface plasmon resonance (LSPR) has been widely employed because of rapid response, reliability and easy-to-make portable sensor [1,2]. LSPR has been used to exploit the affinity of immobilized ligands for detection of their specific targets [3,4]. LSPR-based biosensors can be generated by immobilizing metallic nanostructures onto a substrate that is sensitive to changes in refractive index [4,5]. It is important to note that the binding of targets onto an LSPR sensor chip correlates with peak shifts in LSPR signal, which is dependent on the number of molecules bound to the LSPR sensor layer. LSPR sensor shows fast response with a short decay length in the electromagnetic field, and detects various analytes on its surface with high sensitivity [3-7].

Norovirus is one of the major infectious agents of the gastrointestinal tract, and is a major public health problem worldwide [8-10]. Human norovirus is highly contagious with fewer than 10^2 particles sufficient to cause infections [8,9]. The exposure of norovirus occurs mainly from contaminated foods or other resources including water and infection can rapidly spread from person to person. The majority of infections are caused by GII type [8-10] that is the leading cause of foodborne illness. However, no direct and reliable culture methods of human norovirus in a real test are available [8-10]. This is in part because of the limitations of efficient detection methods. Although there are several methods for detection of norovirus, including enzyme linked immunosorbent assay, reverse transcription polymerase chain reaction, quantitative polymerase chain reaction, and latex agglomeration method [10-14], these techniques are labor intensive with time-consuming step, and require well-trained technicians and expensive analytical instruments [14-19]. Thus, there is a great demand for development of a cost effective and label-free method for the detection of norovirus.

In addition, significant efforts have been made on the fabrication of several analytical detection methods for human norovirus in various food matrices. Briefly, aptamer was incorporated into an electrochemical biosensing platform for sensing of norovirus with enhanced sensitivity [32]. Further, lateral-flow assay and pre-emptive electrochemical [33] techniques have also been developed for immunosensing of norovirus. Peptides modified electrodes have proven to be specific and sensitive biosensor for the detection of pathogenic microorganisms. Since peptides acted as specific recognition elements, we report the development and fabrication of plasmonic sensing device with norovirus binding peptide as a specific biomolecular binder for the selective and sensitive detection of human norovirus.

In this work, we developed an efficient plasmonic biosensor, which is capable of detecting noroviral capsid proteins and human norovirus using gold nanoparticles (AuNPs) chip incorporated with norovirus recognizing affinity peptides. Current immunoassay relies on antibodies as affinity reagents and this is relatively expensive with multiple sample preparation steps [9]. On the contrary, affinity peptide-based detection regimes are becoming increasingly over antibodies for the development of new biosensors [9]. The affinity peptides are relatively small in size and more amenable than antibodies, and can easily attach to the various surfaces of materials for creation of a new biosensor. To the best of our knowledge, this is the first report showing the use of newly identified affinity peptide for detection of human norovirus on a LSPR sensor layer.

Table 1. The sequence of affinity peptides used in this study

Peptide	Sequence
Norovirus binding peptide	QHIMHLPHINTLGGGGSC
Scrambled peptide 1	NHTQKHKPKMKHGGGGSC Used as negative peptide
Scrambled peptide 2	PTKQKHNKHMHKGGGGSC Used as negative peptide

2. Materials and Methods

2.1. Materials

Gold (III) chloride trihydrate ($HAuCl_4$), trisodium citrate, 3-aminopropyl triethoxysilane (APTES), and sodium citrate dehydrate were purchased from Sigma-Aldrich. A glass film for the sensor chip was obtained from CARA Nano Glass Technology (Gumi, Korea). The peptides specific for noroviral capsid proteins and norovirus (GII type) from human stool samples were prepared according to a previous report [20]. We have reported that unique affinity peptides that bind to recombinant noroviral capsid proteins were identified with M13 phage display technique and characterized binding affinities with ELISA. From the results of ELISA, binding affinities of the selected peptides was found to be nanomolar range. The selected affinity peptides were chemically synthesized by Peptron (>95% purity, Daejeon, Korea), and were modified with a C-terminal cysteine for forming a thiol self-assembled monolayer (SAM) and a linker (-GGGGS-) for molecular flexibility, as shown in Table 1. Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Life Technology Inc., USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technology Inc., USA) and 1% penicillin-streptomycin (Gibco, Life Technology Inc., USA) was used to demonstrate the selectivity of affinity peptide towards their targets.

2.2. Preparation of virus samples

Patient fecal samples containing norovirus GII.4 subtype were isolated according to our previous reported [20]. In brief, the samples were mixed with 0.14 M sodium chloride buffer solution containing 0.25 M glycine (pH 7.5) using mixing stomacher and then homogenized. The homogenized sample was centrifuged at 10,000 ×g, 4ºC for 30 min, and its primary supernatant was sequentially transferred into a new centrifugal tube. The precipitate was mixed with 0.14 M sodium chloride buffer solution containing 0.25 M threonine (pH 7.5) and then centrifuged at 10,000 ×g, 4ºC for 30 min. Its secondary supernatant was mixed with the primary supernatant for the further concentration. The mixed supernatant was reacted with 40% (w/v) polyethylene glycol (PEG) and 3 M of sodium chloride, and then incubated at 4ºC for 3 h to obtain the primary precipitate. Next, the sample was centrifuged at 10,000 ×g, 4ºC for 20 min. Its

precipitate was dissolved with 50 mM Tris-HCl containing 0.2% (w/v) of Tween 80 and thoroughly mixed with chloroform: isoamyl alcohol (24:1) solution. The sample was centrifuged at 10,000 ×g, 4ºC for 30 min one more time, and its supernatant was transferred to a new centrifugal tube. The sample was mixed with chloroform: isoamyl alcohol solution and centrifuged one more time, and then the supernatant were added with the previous prepared supernatant. The final mixed supernatant was mixed with 40% (w/v) PEG and 3 M of sodium chloride, and then incubated at 4ºC for 3 h to obtain the secondary precipitate. Finally, the sample was centrifuged at 16,000 ×g, 4ºC for 30 min and the supernatant was removed. Its final precipitate was dissolved with diethyl pyrocarbonate-treated nuclease-free water. More detailed virus preparations including quality test by RT-PCR were described in a previous report [20].

2.3. Spectroscopic analysis

An ultrasonic cleaner set (WUC-A06H, DAHAN Scientific Co. Ltd, Korea) was used to generate inert conditions for immobilizing colloidal AuNPs on a glass substrate. UV–Vis-NIR spectra were recorded using a double beam Jasco V-770 UV–vis-NIR spectrometer (JASCO, USA).

2.4. Electron microscopic analysis

Morphology of the substrate was examined using a HITACHI S-4300 scanning electron microscope (SEM). Transmission electron microscope (TEM, JEM2100F, JEOL Ltd., Japan) was used to examine the binding interaction between affinity peptide and norovirus.

2.5. Synthesis of gold nanoparticles

Gold nanoparticles were prepared according to a previous report [21]. In a typical experiment, 2 mM sodium citrate solution (50 mL) was heated at 90°C using a mantle in a 100 mL three-necked round-bottomed flask (with condenser) for 15 min while stirring vigorously. After boiling, 1 mL of $HAuCl_4$ (20 mM) was injected into the solution. The color of the solution changed from yellow to bluish gray and then to soft pink in 10 min. The resulting particles are coated with negatively charged citrate ions, and hence are well suspended in distilled water.

2.6. Fabrication of LSPR chip

To fabricate the LSPR sensor, a glass substrate was immersed in methanol and sonicated for 20 min to remove debris from the surface. The pre-treated glass was then washed three times with distilled water to completely remove the methanol, followed by soaking in 0.5% APTES solution for 1 h at 50°C. During this procedure, the glass substrate was coated with APTES, and the coated glass substrate was repeatedly washed with distilled water to remove unbound APTES. Next, the APTES-coated glass was immersed in a gold colloidal solution with an average particle-size of 16–18 nm for 12 h. During this process, gold nanoparticles (AuNPs) were immobilized on the APTES-coated glass substrate (≈ 50 particles/200×200 nm). Then, the glass substrate was washed several times with deionized water. At the end of the process, a burgundy colored AuNPs-immobilized glass substrate was obtained. The concentration of synthesized AuNPs can be calculated using the following equation [22];

$$N = \frac{\pi \rho D^3}{6M} \quad \text{(Eqn. 1)}$$

$$C_{Au} = \frac{n}{N} \quad \text{(Eqn. 2)}$$

where N is the number of Au atoms per NP, ρ is the density of fcc Au at 193 g cm^{-1}, M is Au's atomic weight of 197 $gmol^{-1}$, D is the core diameter, and n refers to the precursor molar concentration.

2.7. Optimization of peptide concentration conjugation with LSPR chip

To confirm the optimized conjugation between AuNPs and affinity peptides, different concentration (100 pg/mL – 500 μg/mL) of selected peptides capable for binding with noroviral capsid proteins were reacted with the fabricated AuNPs-coated glass substrate at room temperature for 10 min. Optimization was performed by measuring changes in the refractive index of the AuNPs-immobilized glass substrate modified with the peptides before and after reaction with noroviral capsid proteins at a wavelength of 530 nm using a UV-vis spectrometer.

2.8. Effect of interference on binding interactions

DMEM supplemented with FBS was used to confirm that the peptides can specifically bind to noroviral capsid proteins under the sample matrix. Initially, 10 μg/mL of the norovirus binding peptides was coupled with an AuNPs-coated glass chip. Next, different concentrations ranging from 0.00011 to 100 μg/mL of noroviral capsid proteins were reacted with the LSPR chip at room temperature for 10 min. The change in the refractive index upon binding to noroviral capsid proteins and human norovirus was confirmed using the UV-vis spectrometer at λ= 530 nm.

3. Results and Discussion

3.1. Fabrication of affinity peptide-assisted plasmonic biosensor

Previously, our group reported the use of affinity peptides

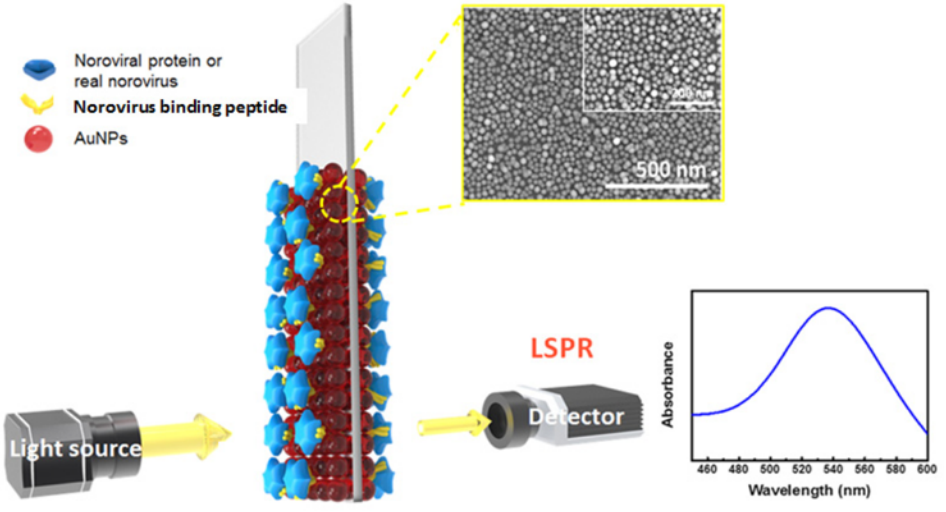
derived from biopanning with polyvalent M13 peptide library [20]. In that study, norovirus binding peptides were incorporated into an electrochemical biosensor with cyclic voltammetry (CV) and quartz crystal microbalance (QCM) techniques for detection of norovirus. From the facts in that study, we postulated that norovirus binding peptides could be used for the development of a plasmonic LSPR biosensor for biosensing of norovirus. The principle of detection was on the bases of LSPR signals for peak shifts those are strongly dependent and varied on the binding and concentrations (lower and higher) of noroviral protein or norovirus on peptide-tethered LSPR sensor chip. To test our hypothesis, we tried to fabricate a plasmonic-based biosensor system. To do this, AuNPs were synthesized according to the previously reported procedure [21]. Fig. S1 shows TEM image and UV-vis spectrum of the synthesized AuNPs. TEM image clearly showed that the AuNPs were well-dispersed with an average particle-size < 20 nm (Fig. S1a, Electronic Supplementary Material). Surface plasmon absorption spectrum of AuNPs showed a maximum adsorption peak at 523 nm (Fig. S1b, Electronic Supplementary Material). The synthesized AuNPs were show to be immobilized on a surface of APTES-functionalized glass substrate. Pre- and post-immobilization of AuNPs on glass substrate images are shown in Fig. S2, Electronic Supplementary Material. The concentration of synthesized AuNPs can be calculated using the equation 1 and 2 (Eqn. 1 and 2), as previously mentioned above.

We measured that the final concentration of AuNPs was 1.78×10^8 NPs/mL in triplicate and the efficiency of recovery was quite reproducible. Based on these findings, we wanted to develop a method for LSPR plasmonic biosensor for detection of noroviral capsid proteins and norovirus using specific interaction between norovirus binding peptides and their targets. Therefore, norovirus binding peptides were used as recognition reagents to detect norovirus capsid proteins and human norovirus on the plasmonic active substrate that was fabricated by immobilization of gold nanoparticles onto APTES-functionalized glass substrate (Scheme 1). We believe that the fabricated plasmonic biosensor system can be used straightforward detection tools for monitoring noroviral proteins and human norovirus. Further experimental characterization of the proposed sensor system will be described in the following section.

3.2. Characterization of plasmonic LSPR biosensor

To evaluate the functionalized plasmonic substrate, we varied the concentration of norovirus binding peptides on a plasmonic substrate layer. To optimize binding of cysteine-incorporated peptides on the AuNPs-glass substrate, norovirus binding peptides at concentrations ranging from 0.001 to 100 μg/mL were tested. Fig. 1A shows that relative binding to the AuNPs-glass chip is proportional with increasing concentration of norovirus binding peptides. The absorbance changed linearly up to 10 μg/mL of norovirus binding peptides, saturating around concentrations of 100 μg/mL which may be due to overloading of norovirus binding peptides. At norovirus binding peptide concentrations higher than 100 μg/mL, there was no further significant increase in the LSPR signal. Therefore, we determined 10 μg/mL of norovirus binding peptides to be useful for plasmonic-based sensor system.

Next, the concentrations of noroviral capsid proteins were varied with 10-fold serial dilution from 0.0001 to 100 μg/mL and reacted with AuNPs-coated glass chip



Scheme 1. Schematic illustration of norovirus binding peptide-guided plasmonic biosensor for detection of norovirus capsid proteins and human norovirus.

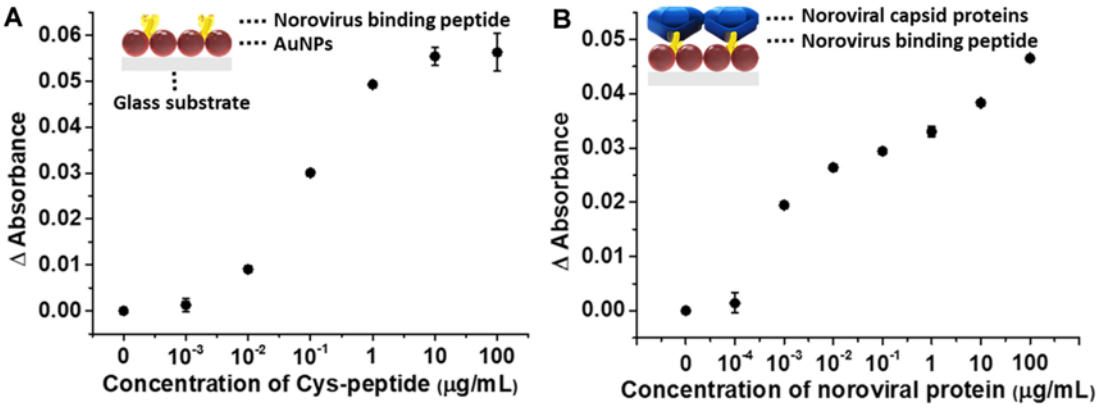


Fig. 1. Effect of norovirus binding peptides (A) and noroviral capisid proteins (B) on binding interactions.

conjugated with norovirus binding peptides. It was found that the absorbance gradually increased as the concentration of noroviral capsid proteins increased (Fig. 1B). These results showed that norovirus binding peptides can be detected at concentrations as low as 1 ng/mL using LSPR.

3.3. Effect of interference (DMEM and FBS) on binding interactions

The binding of norovirus binding peptides to noroviral capsid proteins was measured by changes in absorbance in the presence of both DMEM and FBS. Fig. 2 shows the plot of change in absorbance with respect to spiked noroviral capsid proteins in the range of 0.0001 to 100 μg/mL under sample matrix (DMEM+FBS) conditions. As shown in Fig. 2, change in the absorbance increased proportionally with increasing concentration of the noroviral capsid proteins. A dynamic increase in the absorbance was directly related to the interaction between norovirus binding peptides and noroviral capsid proteins. As expected, no the change of absorbance on norovirus binding peptide-immoblized sensor layer without addition of noroviral capsid proteins is observed in the same condition. A steady change in absorbance was observed above concentration of 10 μg/mL, indicating that the binding interaction between noroviral capsid proteins and norovirus binding peptides saturated in the plot. Based on these results, we inferred that specific binding of noroviral capsid protein is possible with the norovirus binding peptide at 10 μg/mL concentrations, in presence of interference from the co-existing sample matrix in the medium.

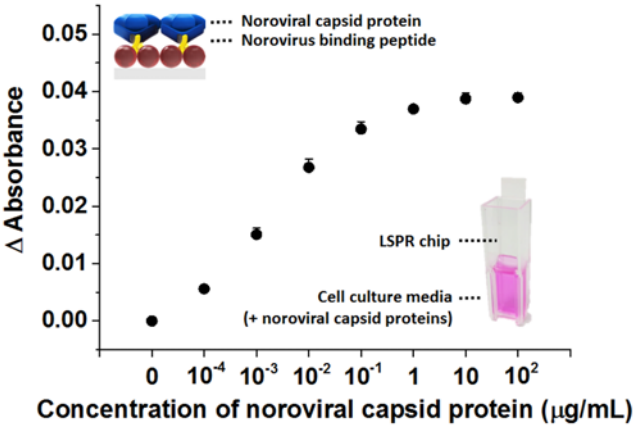


Fig. 2. Effect of interference (DMEM+FBS) on binding interactions. Different concentrations ranging from 0.00011 to 100 μg/mL of noroviral capsid proteins were reacted with the LSPR chip at room temperature for 10 min. The change in the refractive index upon binding to noroviral capsid proteins was confirmed using the UV-vis spectrometer.

3.4. Validation of plasmonic biosensor

In order to validate plasmonic biosensor for detection of human norovirus, LSPR signals were monitored by varying the concentrations of human norovirus (10^1 to 10^5 copy/mL)

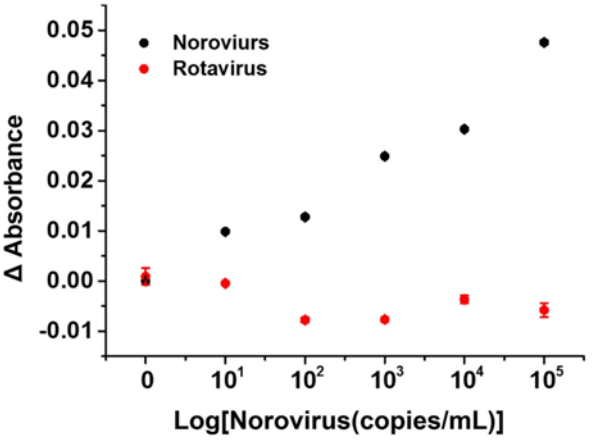


Fig. 3. Selectivity test of plasmonic sensor for detection of human norovirus and rotavirus.

from fecal samples and rotavirus. Fig. 3 shows that the absorbance of LSPR increased linearly with increasing concentration of norovirus between the wide range of norovirus concentrations and LSPR signal, while no distinct changes in the LSPR signal in the presence of rotavirus. However, no further significant difference in LSPR absorbance was observed with scrambled peptide 1 and 2 as negative control (Fig. S3, Electronic Supplementary Material). In addition, we observed the binding of human norovirus to immobilized peptide by TEM (Fig. S4, Electronic Supplementary Material). As expected, norovirus binding peptides can be bound to human norovirus. It was suggested that norovirus binding peptide-assisted plasmonic sensor was highly specific and selective for norovirus, not rotavirus.

3.5. Performance of plasmonic biosensor

To further validate the sensor system, the change of absorbance in LSPR was also monitored by varying the concentrations of human norovirus. We observed dynamic response with increasing concentration of norovirus (Fig. 4). The limit of detection (LOD) of our sensor was found to be 9.9 copies/mL by the two-segment linear relationship (3σ/slope) [23], as shown in Fig. 4. LOD values on this sensor system are much lower than those observed for other detection approaches, including ELISA (4.2×10^8) [24], commercial LFA (10^6-10^7) [25,26] and RT-PCR (1.6×10^7) [27]. The comparison of the performance of detection was shown in Table S1, Electronic Supplementary Material.

Previous studies [28-31] indicates that affinity peptides derived from phage display are robust and easy to incorporate with other recognition reagents as molecular probe, such as biomolecules and nanoparticles. In addition, the use of affinity peptides is fascinating because (i) mass production of peptides is relatively cost-effective than antibody production, (ii) affinity peptides identified by using the phage display technique are smaller than antibodies, and (iii) free peptides separated from phage particles can be easily synthesized to create peptide libraries for developing biosensors. Most interestingly, affinity peptides are either linear or in a cyclic form, indicating that peptides are more amenable than antibodies to engineering at the molecular level. Based on these observations, this general strategy is straightforward and can be used for highly sensitive and label-free norovirus detection in contaminated food samples.

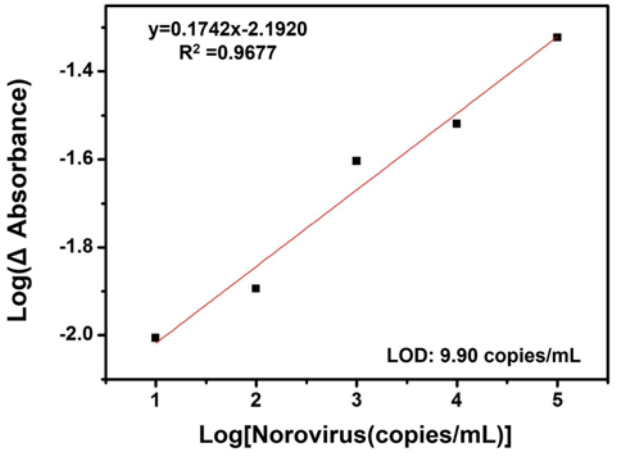


Fig. 4. The performance of plasmonic sensor for detection of human norovirus.

4. Conclusion

In summary, we have successfully developed an effective and rapid bioanalytical method through norovirus binding peptide-guided plasmonic biosensor using LSPR techniques. LSPR signals quantitatively detected response to noroviral capsid proteins and human norovirus, and thus can be used for highly sensitive detection. It is noteworthy that LOD value of this sensor system are acceptable for norovirus-related protein or human norovirus detection. This approach is straightforward and did not require any pre-concentration steps. Further validation of the sensor with real clinical samples is currently in progress. The approach described herein will enable development of norovirus binder-guided bioanalytical platform as a monitoring tool for detection of desired targets or other pathogenic bacteria. This novel peptide coated LSPR biosensor provides a simple, inexpensive and rapid analytical strategy to detect human norovirus, which offers a promising strategy for identification and quantification of norovirus in food contaminants and clinical samples with minimized sample preparations and volumes. The developed LSPR glass devices may open new horizons in the advanced field of novel biosensors for point-of-care viral infection detection.

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