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A rapid, sensitive and selective electrochemical biosensor with concanavalin A for the preemptive detection of norovirus

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ABSTRACT

Norovirus (NoV) is a foodborne pathogen that can cause sporadic and epidemic gastrointestinal diseases. Rapid screening is crucial to promptly identify the presence of NoV and prevent food poisoning. Here, we present a sensitive, selective, and rapid electrochemical biosensor for the detection of NoV. The proposed electrochemical biosensor is composed of a nanostructured gold electrode conjugated with concanavalin A (ConA). ConA functions as a recognition element that selectively captures NoV. Cyclic voltammetry revealed a linear relationship (R^2=0.998) between the current and concentration of NoV (in the range of 10^2 and 10^6 copies/mL), with a relatively short assay time (1 h) and a good detection limit (35 copies/mL). Additionally, the signals of Hepatitis A and E in the selectively test were found to be only 2.0% and 2.8% of the NoV signal at an identical concentration of 10^3 copies/mL, proving that the electrochemical biosensor has a selectively of approximately 98%. Moreover, the concentration of NoV was measured in a realistic environment, i.e., a sample solution extracted from lettuce, to demonstrate a potential application of the proposed biosensor (LoD=60 copies/mL).

1. Introduction

It is known that Norovirus (NoV) causes more than 90% of reported outbreaks of nonbacterial gastroenteritits in the United States (Koo et al., 2010; Patel et al., 2009). NoV is an RNA virus, which falls into the category of Caliciviridae viruses, is classified into five genogroups (e.g., GI to GV) by polymerase and capsid protein sequencing; to date, 29 known genotypes are known. Among these, eight genotypes in GI and seventeen genotypes in GII are known to cause infection in humans (Palchetti and Mascini, 2008). According to the recent studies carried out by the U.S. Center for Disease Control and Prevention (CDC), above 70% of waterborne and 50% of foodborne of cases of enteritis in the United States were caused by NoV (Patel et al., 2009). Infection by NoV is possible even with virus amounts smaller than 10^2 copies/mL. Prevention is, however, hampered by the strong resistance of NoV to acid, heat (60 °C), and to chlorine concentrations in tap water (3.75–6.25 mg/L) (Lee et al., 2013). More than 50% of infections are known to occur in public spaces, such as restaurants, school cafeterias, and cruise ships. NoV infections are possible via direct infection, person-to-person secondary infection, or spreading by food handlers (Sharma and Mutharasan, 2013; Hall et al., 2011; Atmar et al., 1995; Schwab et al., 2000; Seymour and Appleton, 2001; David et al., 2007; Fino and Kniel, 2008; Friedman et al., 2005; Gaulin et al., 1999). The symptoms of foodborne illnesses generated by NoV include vomiting, gastralgia, diarrhea, fever and, rarely, death of the elderly or children due to dehydration (Daniels et al., 2000; Glass et al., 2001; Tian and Mandrell, 2006).

To date, detection of NoV is typically carried out on suspected food after patients report gastroenteritis symptoms (Kim et al., 2011) with a range of methods, including Enzyme-Linked Immunosorbent Assay (ELISA) (Jiang et al., 1992), Western Blot (Hayashi et al., 1989), Reverse Transcription Polymerase Chain Reaction (RT-PCR) (Vinje et al., 2003; Guyader et al., 1996), Real-time RT-PCR (Kageyama et al., 2003), and the rapid antigen test (QuickNavi-Noro kit; Denka Seiken Co., Tokyo, Japan) (Kim et al., 2011). Among these, RT-PCR and Real-time RT-PCR are considered as the most accurate methods in terms of sensitivity ($< 10^2$ copies/mL). However, PCR requires relatively expensive equipment and a well-trained technician; furthermore, in order to obtain a final diagnosis, a considerable amount of time and high costs are needed ([Flekna et al., 2007,D'Souza and Jaykus, 2002,Kim et al., 2008,

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Sobsey et al., 1978]). Notably, in cases, in which NoV could be preemptively identified, the infection was largely blocked in public places (Sharma and Mutharasan, 2013; Hall et al., 2011; Atmar et al., 1995; Schwab et al., 2000; Seymour and Appleton, 2001; David et al., 2007; Fino and Kniel, 2008; Friedman et al., 2005; Gaulin et al., 1999). Therefore, research has aimed at developing preemptive, rapid and efficient methods to be applied in public spaces. To date, these include an antigen method that employs test strips (QuickNavi-Noro kit; Denka Seiken Co., Tokyo, Japan) (Kim et al., 2011) and an electrochemical biosensing method, based on ELISA and electrochemistry (Giamberardino et al., 2013). Rapid antigen tests are based on immune chromatography and detect NoV antigens in food via the immobilization of NoV antibodies on the device. The advantages of this approach include inexpensiveness and facile on-site application without pretreatment; however, the rapid antigen tests are characterized by poor sensitivity and show scarce reproducibility. In contrast, electrochemical biosensors have advantages over analytical transducing systems, such as fast detection, good quantification, relatively high sensitivities (10^{-7}–10^{-9} M), ease of data logging, and the possibility of miniaturization (Palchetti and Mascini, 2008; Ivnitski et al., 1999; Leonard et al., 2003; Patel, 2002; Wei et al., 2014; Mandal et al., 2014; Sha et al., 2014; Zhang et al., 2014; Luciana et al., 2004; Almira et al., 2012). In addition, highly sensitive sensors can be introduced over large surface areas within the same electrode using electrochemical deposition (Evans-Nguyen et al., 2008). Recently, a NoV aptamer electrochemical sensor was reported to detect human NoV via a label-free method, with a short assay time (1 h) and 180 copies/mL limit of detection (LoD) (Giamberardino et al., 2013). Typically, label-free methods are simpler than those methods that require labeling, however, at the expense of sensitivity. More generally, electrochemical biosensors based on antibody–antigen reactions show good selectivity and sensitivity, but, because of the high price, they are not suitable to be used in disposable chips for point-of-care tests (POCT) (Sano et al., 2004; Ko et al., 2014). In this contribution, we introduce an alternative capturing agent for the electrochemical sensor applications, which, besides good sensitivity and selectively, is relatively cheap (1/50 times cheap compared with using antibody) and can detect NoV in a shorter amount of time (1 h).

The capturing agent proposed in this work is concanavalin A (ConA), a lectin (carbohydrate-binding protein) originally extracted from *Canavalia ensiformis* (jack bean). ConA is able to bind sugars, glycoproteins, and glycolipids, such as internal and non-reducing terminal α-D-mannosyl and α-D-glucosyl groups (Lis and Sharon, 1986; Bittiger and Schnebli, 1976). ConA is known to bind with some of bacterias such as *Escherichiacoli* and *Bacillus subtilis* and protist *Dictyostelium discoideum* (Jonathan and Catherine, 1999; Christopher and Daniel, 1977; Doyle and Birdsell, 1972). Also, ConA can bind to a broader range of viruses such as varicella-zoster virus, avian influenza virus, dengue virus, human immunodeficiency virus, and infectious bronchitis virus (Yokoyama et al., 2001; Fu et al., 2014; Schieffelin et al., 2010; Kim et al., 2009; Bronzoni et al., 2005). Notably, ConA can selectively capture NoV unlike other foodborne viruses, such as those of hepatitis A (HAV) and hepatitis E (HEV) (Kwon et al., 2013). In the electrochemical biosensor proposed in this work, ConA was placed on a nanostructured gold electrode. The detection and secondary antibodies were immobilized and conjugated in series with alkaline phosphatase (ALP), an electrochemical enzyme. The gold-black electrochemical deposition method was used to create a nanostructured large surface area on the plain gold surface in order to maximize the surface area, thereby yielding the maximized the signal in surface binding assay (Blake et al., 2013). The electrochemical biosensor developed in this work represents a straightforward approach to detect NoV in optically dense or turbid samples (such as extracted solutions from lettuce) with a low LoD, good reproducibility and stability.

2. Materials and methods

2.1. Materials and reagents

Mercaptohexanol (MCH), gold(III) chloride hydrate 99.999%, ConA, protease inhibitor cocktail, and anti-rabbit IgG-ALP were purchased from Sigma (St. Louis, USA); 4-aminophenylphosphate monosodium salt hydrate (APP) was obtained from gold biotechnology (St. Louis, USA). Skim milk protein was purchased from Lab M limited (Lancashire, UK). Rabbit polyclonal anti-NoV antibody was obtained from Abcam (Cambridge, UK). All chemicals and solvents used in this work were of analytical grade and were used as received. All buffer solutions were prepared in Milli-Q water (Millipore, USA). The buffer solutions employed in this work were as follows: Reaction buffer for APP: 50 mM Tris–HCl (pH 9.6) containing 10 mM $MgCl_2$; Buffer for other chemicals: Tris-buffered saline (1 × TBS); Rinse buffer used to remove all non-specific binding: 1 × TBS with 0.05% Tween.

2.2. Preparation of virus samples

Patient fecal samples containing NoV GII-4 subtype and HEV viruses were obtained from Gwangju Health and Environment Research Institute and they had been isolated. HAV strain HM-175/18f (VR-1402) was obtained from the American Type Culture Collection (Manassas, VA) and was propagated in fetal rhesus kidney (FRhK-4) cells. The quality of each type of viruses were confirmed by RT-PCR and the detailed information could be found in the previous report.

2.3. Preparation of samples extracted from lettuce

The lettuce extraction was conducted by a procedure modified from a method previously described by Kingsley and Richards (Kingsley and Richards, 2001). Firstly, 10 g of crisphead lettuce was homogenized in 50 mL of Tris elution buffer (100 mM Tris–HCl, 50 mM glycine, 1% beef extract, pH = 9.5) at room temperate using a stomacher (LB-400, Sibata, Japan) for 15 min. Then, a prepared sample was centrifuged at 15,000*g* for 10 min at 4 °C in order to remove the lettuce pellet; only the supernatant was collected. Seven samples were prepared with various concentrations of NoV, e.g., 0 (blank), 10^1, 10^2, 10^3, 10^4, 10^5, and 10^6 copies/mL.

2.4. Fabrication of the nanostructured gold electrode

The gold electrode was fabricated using a typical microfabrication process. The positive photoresist (GXR-601) was patterned on Au(300 nm)/Cr(30 nm) film sputtered on a glass substrate. After lithography process, the glass substrate was etched in etchants (Sigma-Aldrich, St. Louis, MO, USA) to remove Au and Cr. The polydimethlysiloxane (PDMS) micro-well used to contain the buffer sample was made with a 6 mm punch. The bonding between the glass (bottom substrate) and the PDMS block was achieved via an O_2 plasma process. The prepared gold electrode was cleaned in acetone with sonication (15 min), methanol (10 min), and Deionized water (DI water) (10 min). After rinsing the electrode, the excess solvents were blown off under a stream of nitrogen. To make the nanostructured gold electrode, a gold solution was prepared for the electrochemical deposition as shown in Fig. S1. The nanostructured gold electrode was produced as described previously (Blake et al., 2013). In particular, 3 mg/mL gold(III) chloride hydrates were immersed in 0.5 M H_2SO_4. In order

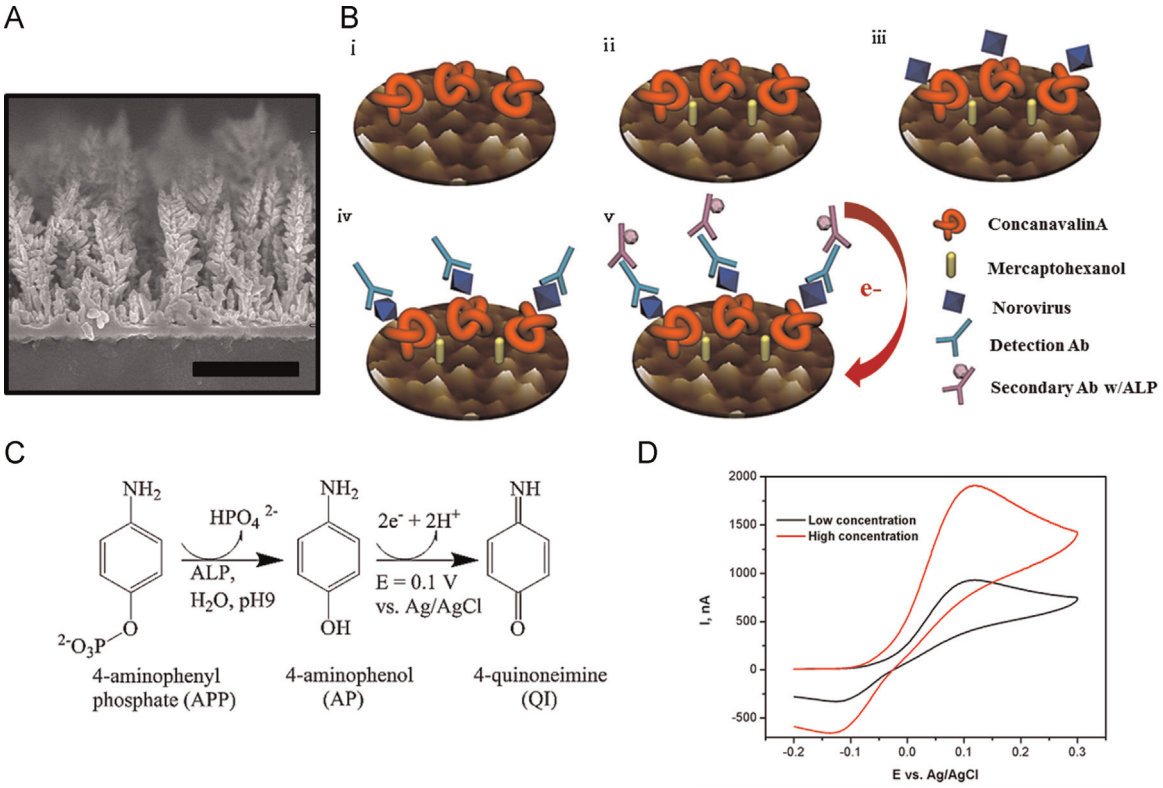

Fig. 1. Preparation and characterization of the electrochemical biosensor. (A) The scanning electron microscope (SEM) image was obtained using a Hitachi S4700; the scale bar is 2 μm. (B) Schematic representation of the proposed electrochemical sensor: (i) after immobilization of ConA, (ii) after blocking by MCH, (iii) after immobilization of norovirus, (iv) after immobilization of detection Ab, and (v) after immobilization of secondary Ab with ALP. (C) ALP-labeled antibody converts APP to AP, which is electrochemically oxidized to generate a current at the electrode that is proportional to the amount of NoV bound to the sensor surface. (D) Signal is read using cyclic voltammetry (CV).

to induce the electrochemical deposition of the nanostructured Au, a potential of −400 mV was applied to a plain gold film (working electrode – 1.5 mm diameter) against an Ag/AgCl reference electrode under controlled stirring. In previous studies (Blake et al., 2013), with the aim of optimizing the gold-black deposition time, surface area, cyclic voltammetry (CV) data, and non-contact atomic force microscope (AFM, Park Systems XE-100 using a PPP-NCHR 10 M noncontact cantilever tip) data were introduced with respect to electrochemical deposition time. The optimized deposition time, i.e., the minimum deposition time required to obtain the maximum surface area, was around 400 s. After deposition, the nanostructured gold electrode was rinsed thoroughly, blown off under a nitrogen stream, and stored in a sealed petri dish. The scanning electron microscope (SEM) image was obtained using a Hitachi S4700; the scale bar is 2 μm (Fig. 1(A)).

2.5. Electrochemical measurements

All electrochemical experiments, including CV and electrochemical impedance spectroscopy (EIS), were performed on an electrochemical analyzer (CHI760D, CH Instruments) at room temperature. Electrochemical measurements were carried out using a three-electrode system consisting of the nanostructured gold electrode as the working electrode, an Ag/AgCl (sat. KCl) reference electrode, and a platinum counter-electrode. CV and EIS measurements were used to monitor the stepwise fabrication of the biosensor. CV measurements were carried out with a scanning rate of 50 mV/s between −0.2 and +0.7 V relative to the Ag/AgCl reference electrode. EIS measurements were performed at a frequency range between 0.1 and 100 kHz with signal amplitude of 5 mV. Data were collected in the presence of a Tris–HCl buffer (2.5 mM Fe $(CN)_6^{4-/3-}$ +0.1 M KCl, pH 7.4).

2.6. Preparation of NoV biosensor

A schematic representation of the working principle of the proposed biosensor is illustrated in Fig. 1(B). A 100 μg/mL solution of ConA in TBS was drop cast on the nanostructured gold electrode for 1 h at 4 °C. After washing step, the ConA-immobilized electrode was further treated with 30 μL of drop-cast 1.0 mM mercaptohexanol (MCH) for 2 h at 25 °C and was subsequently washed with DI water. A pretreated electrode was incubated with six concentrations of NoV, ranging from 10^1 copies/mL to 10^6 copies/mL in TBS at 4 °C. After immobilization, the electrode was blocked with 30 μL of blocking buffer (1% dehydrated milk in TBS) at 4 °C. For the immobilization of the detection antibody, the electrode was immersed in 10 μg/mL rabbit polyclonal anti-NoV antibody in 10 μL of 0.5% milk in TBS at 4 °C. After washing, the electrode was then incubated in a solution of anti-rabbit IgG-ALP at 1 μg/mL prepared in 0.5% milk in TBS at 4 °C. The electrode was extensively rinsed with washing buffer (1 × TBS+0.05% Tween). Electrochemical measurements for NoV sensing were carried out at room temperature and the potential swept from −0.2 V to +0.3 V with a scan rate of 20 mV/s in a 50 mM Tris–HCl (pH 9.6) with 10 mM $MgCl_2$ containing 20 mM APP. As shown in Fig. 1(C), APP was converted to aminophenol (AP) by ALP on the electrode, and then electrochemical signal was obtained when AP was oxidized to quinoneimine (QI).

The electrochemical signals related to the target concentration captured by anti-rabbit IgG conjugated ALP on the electrode were measured (Fig. 1(D)).

3. Results and discussion

3.1. Characterization of the NoV biosensor

The immobilization of the agents on the electrode surface was confirmed at each step. CV and EIS were used to characterize the nanostructured gold electrode surface in the presence of a $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ redox probe. The redox probe $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ revealed a reversible CV at the nanostructured gold electrode (Fig. 2(A), curve a). After the nanostructured gold electrode was immobilized with ConA, the penetration of the redox probe was hampered and the current decreased (curve b). After the electrode was modified with MCH, the redox probe penetrated the electrode surface, thereby decreasing the current (curve c). When NoV was captured by the immobilized ConA on the electrode, the current decreased again (curve d). After immobilization of the detection antibody and ALP-labeled secondary antibody, the penetration of the redox probe decreased further (curves e, f). In addition, the impedance spectra exhibited a semicircle at higher frequencies depicting the electron transfer limited process and a linear portion at lower frequencies implying diffusion process (Fig. 2(B)). The Nyquist plot of the bare nanostructured gold electrode was found to be nearly linear, indicating that the transfer or charges were completely unhindered (200 Ω, curve a). After immobilization of ConA, the diameter of the semicircle in the Nyquist plots increased with the resistance (505 Ω, curve b), possibly because ConA hindered electron transfer at the electrode interface. After blocking with MCH, the diameter of the semicircle increased (1010 Ω, curve c). As expected, when NoV was selectively bounded to the immobilized ConA, the penetration of the redox probe was reduced further, resulting in a higher electron transfer resistance (R_{et}) value (1140 Ω, curve d). After immobilization of the detection antibody and the ALP-labeled secondary antibody, the resistance increased further, indicating interactions between NoV and the detection antibody (1200 Ω, curve e) and between the detection antibody and the secondary antibody (1250 Ω, curve f).

Thus, these results confirmed that the immobilization of individual agents at each step has been successfully conducted.

3.2. Detection of the NoV in TBS

To detect the virus, the electrical current of TBS solutions with various NoV concentrations (0 (blank), 10^1, 10^2, 10^3, 10^4, 10^5, and 10^6 copies/mL), was measured as shown in Fig. 3. The volume of TBS solution with various concentrations of norovirus was 100 μL. We would like to remark that the criterion for NoV infection is about 10^2 copies/mL in concentration (Patel et al., 2009). The CV plot in Fig. 3(A) shows that the oxidation currents increase with respect to the elevated NoV concentration, which in turn implies that ALP increases with the concentration of NoV. The difference in measured current between curve a and b is almost zero with both 0 and 10^1 copies/mL in NoV concentration. From 10^2 copies/mL, however, distinct changes in the electrical current appear with an increasing concentration of NoV (curves c–g). This finding indicated that the LoD of the proposed biosensor is about 10^1 copies/mL. Fig. 3(B) shows the linear relationship between the changes in electrical current and the NoV concentrations (in the range of 10^2 and 10^6 copies/mL), with R^2 being 0.998. The calibration curve was calculated from the current value at a voltage of 0.1 V, which is obtained when AP is oxidized to QI. The LoD is defined as $3 \times (SD/S)$ (Patricia et al., 2014), where SD is the standard deviation of blank and S the slope. The LoD was obtained 35 copies/mL with less than 5% in relative standard deviation (RSD) for the NoV concentration range between 10^2 and 10^6 copies/mL ($n=5$).

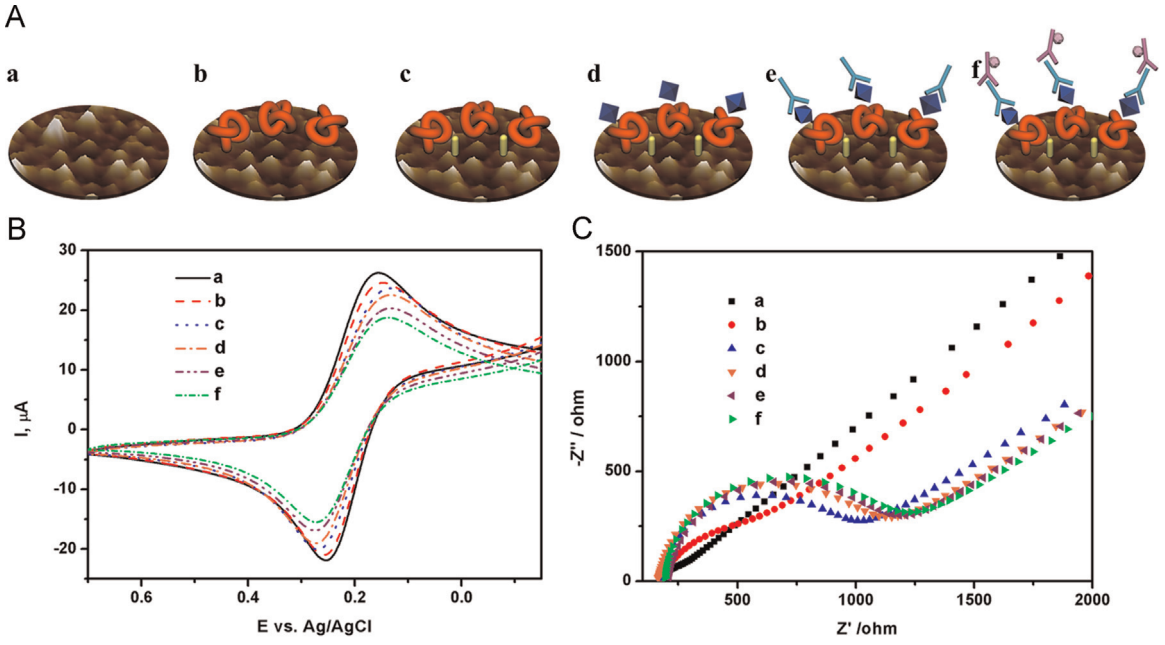

Fig. 2. (A) Schematic representation of the proposed electrochemical sensor (B) CV, and (C) Nyquist plots after the assembly of the nanostructured gold electrode in the presence of 2.5 mM Fe $(CN)_6^{4-/3-}$ +0.1 M KCl, pH=7.4 (scan rate of 50 mv/s). (a) The nanostructured gold electrode; (b) after immobilization of ConA; (c) blocking by MCH; (d) after immobilization of NoV; (e) after immobilization of detection Ab; (f) after immobilization of secondary Ab with ALP.

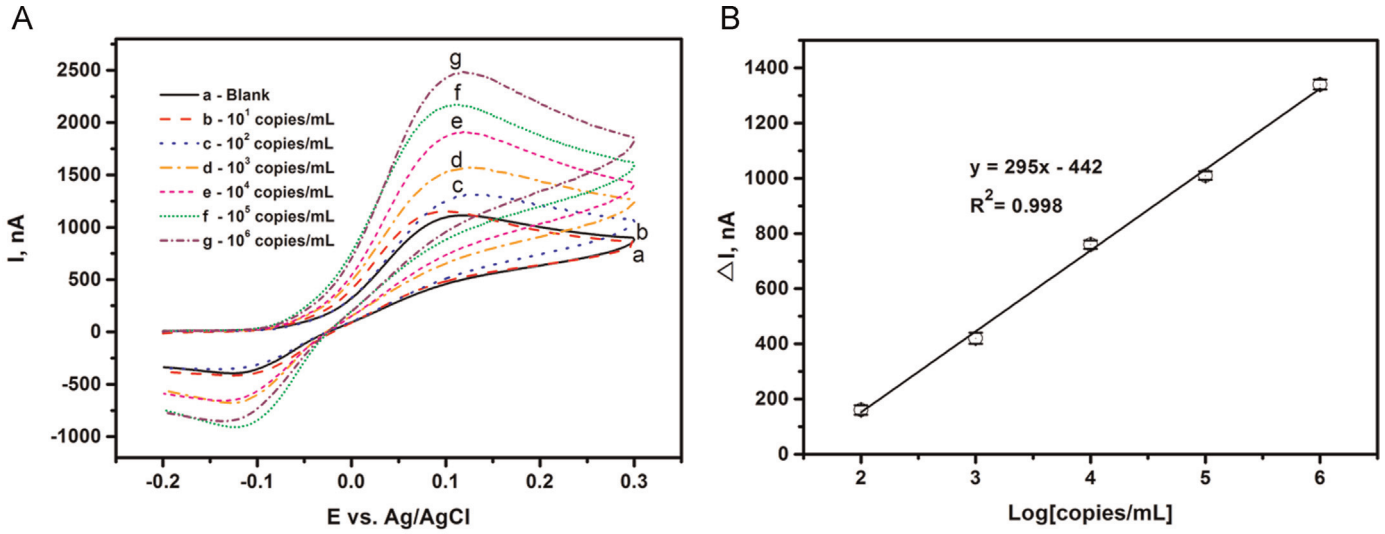

Fig. 3. (A) Cyclic voltammetry measurements at −0.2 V to 0.3 V vs. Ag/AgCl for different NoV concentrations; (B) linear relationship between the logarithmic value of the NoV concentration and the ▵current ($Current_{NoV} - Current_{Blank}$; where $Current_{NoV}$ is the current in the presence of NoV and $Current_{Blank}$ is the current in the absence of NoV) at 0.1 V, which corresponds to the oxidation of AP.

3.3. Selectively of the NoV biosensor

In our test, both HAV and HEV with 10^3 copies/mL in concentration were used in the presence of NoV (10^3 copies/mL). As a result, the electrical currents for HAV and HEV were measured as 14.67 nA and 19.9 nA, which are 2.0% and 2.78% in non-specific reaction compared with the current value from the NoV detection test (715 nA) at the same concentration level. An additional statistical test (*t*-test) has been conducted to check whether the current signals obtained from each sample are statistically different by assuming that the two sample sets have equal variances. As shown in Fig. 4 below, the *p* Value obtained from the *t*-test between the NoV and the HAV is below 0.05 and also it is below 0.05 for the NoV and HEV. Thus it is clear to say that the 2.0% and 2.78% signal intensities of the HAV and HEV are statistically negligible ones compared with the one obtained from the NoV sample. Thus, it is believed that the proposed biosensor is stongly selective to NoV.

3.4. Reproducibility and thermal stability of the NoV biosensor

The reproducibility of the proposed biosensor was evaluated by measuring the electrical current of the same concentration of NoV (10^2 copies/mL) with five different electrodes. The mean current was calculated as 1310 nA (RSD equal to 4.38%), implying acceptable reproducibility. In addition, in order to estimate the thermal stability of the sensor, the electrodes were prepared at different temperature (4 °C, 25 °C, 30 °C, 35 °C, and 40 °C) for a week. The electrochemical signals from NoV sample with protease inhibitor, which suppresses the protease activity, were obtained at room temperature. As shown in Fig. S2, the electrical current decrease (in comparison to the current value obtained using same electrode without special storage process) was determined to be as 97.5%, 96.9%, and 68.8% at 4 °C, 25 °C, and 40 °C, respectively. The strong reduction of the current at 40 °C may be due to the degradation of ConA.

3.5. Detection of the NoV in samples extracted from lettuce

In order to demonstrate the capability of the biosensor for practical applications, detection of NoV was carried out using a solution extracted from lettuce (Fig. 5). The entire procedure for NoV detection is illustrated in Fig. 5(A). The NoV samples were detected using the method described above. The volume of lettuce extract

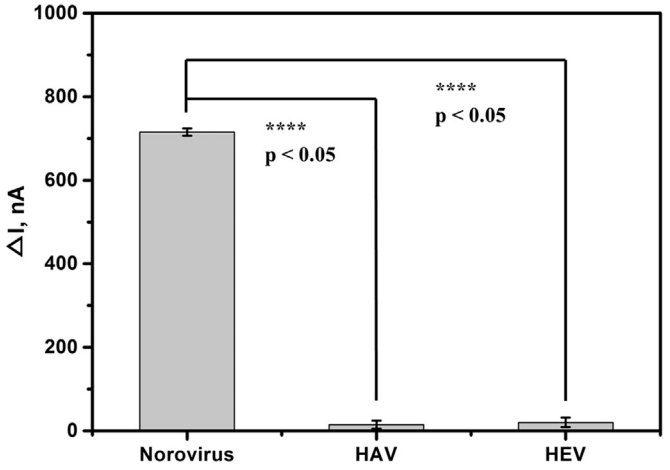

Fig. 4. Investigation of the biosensor selectively. The concentration of the three different viruses was 10^3 copies/mL. ▵Current ($Current_{sample} - Current_{Blank}$; where $Current_{sample}$ is the current in the presence of NoV, HAV, and HEV and $Current_{Blank}$ is the current in the absence of NoV) at 0.1 V, which corresponds to the oxidation of AP.

with various concentrations of norovirus was 100 μL. Unlike for TBS, in the case of the solutions extracted from lettuce, the current value dropped to 70% of the one measured in pure TBS. This was possibly caused by unknown proteins, which could bind either NoV or the surface of the sensor, thereby reducing the binding area. Despite these interferences, the linear relationship between the current and NoV concentration was not affected, the R^2 being 0.968, in the NoV concentration range between 10^2 and 10^6 copies/mL (Fig. 5(B)). Even though the LoD of the sample prepared from lettuce (60 copies/mL) was determined to be higher than that in TBS (35 copies/mL), it was still lower than the criterion for NoV infection (10^2 copies/mL). Therefore, these data clearly showed that the proposed biosensor can be successfully applied for the on-site detection of NoV in food.

4. Conclusions

In this work, we proposed an electrochemical biosensor with a NoV-selective capturing agent (ConA) to be used in preemptive on-site detection of NoV.

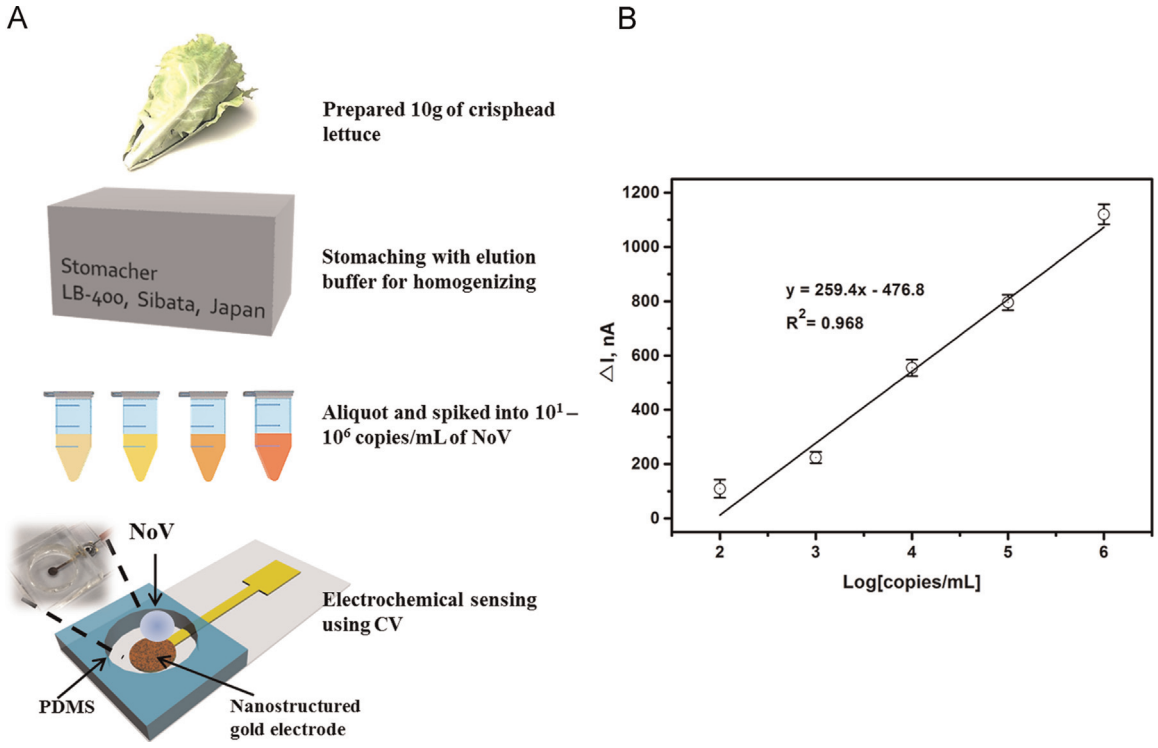

Fig. 5. (A) Procedure of NoV detection in solutions extracted from lettuce. (B) Current in solutions extracted from lettuce inoculated with NoV (concentration ranging between 10^2 and 10^6 copies/mL).

CV and EIS measurements were carried out to characterize the functionality of the biosensor in the presence of $Fe(CN)_6^{3-}$/$Fe(CN)_6^{4-}$ as a redox probe. Our measurements showed that the biosensor could detect NoV in a concentration range of 10^2 and 10^6 copies/mL in TBS; a linear relationship (R^2=0.998) was also established (with 35 copies/mL in LoD within 1 h). In addition, the biosensor was successfully able to detect NoV in the same concentration range in solutions prepared from lettuce (with 60 copies/mL in LoD). Moreover, the biosensor showed good reproducibility (RSD=4.38%) and thermal stability (decrement of 2.5% and 3.1% at 4 °C and 25 °C, respectively).

Thus, our data clearly demonstrated the promising performance of the proposed electrochemical biosensor, which is characterized by high sensitivity and selectivity as well as good reproducibility. We expect the proposed electrochemical biosensor to be employed for a preemptive, rapid, and on-site detection of NoV. Further studies are currently carried out to reduce the assay time and improve the sensitivity of the biosensor.

Acknowledgments

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.09.025.

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