Improvement of Electrochemical Conditions for Detecting Redox Reaction of K₃[Fe(CN)₆] toward the Application in Norovirus Aptasensor

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ABSTRACT
Electrochemical biosensors have attracted significant attention as a novel tool for the sensitive detection of pathogens and contaminants, with the potential capability of rapid, on-site diagnosis. In this study, we intended to improve the sensitivity of electrochemical biosensor using Fe(CN)₆³⁻/⁴⁻ as redox marker, toward its application to norovirus aptasensors. Although many researchers have developed electrochemical aptasensors for various analytes using redox markers, the reported electrochemical conditions under which an aptasensor was examined varied across multiple studies. Here, we performed square-wave voltammetry (SWV) for electrodes modified with aptamer specific to murine norovirus and compared the reduction peak currents of Fe(CN)₆³⁻ under various conditions. Effects of working electrode materials and NaCl and [Ru(NH₃)₆]Cl₃ concentration in the electrolyte were examined. Among conditions we tested, the best sensitivity was obtained using a screen-printed gold electrode in an electrolyte containing 1 M NaCl with 4 mM K₃[Fe(CN)₆], in which the concentration of murine norovirus showed linearity with SWV peak current. This study provided useful information on the electrochemical measurement conditions regarding the electrochemical detection of norovirus aptasensors.

Keywords: Norovirus, Aptamer, Screen-printed Electrode, Biosensor

1. Introduction
Biosensors are analytical devices that rapidly convert biological response into electrical signal, and they have been attracting significant attention as novel tools for the sensitive detection of pathogens and contaminants in clinical and environmental settings, with the potential capability of rapid, on-site diagnosis. A biosensor typically comprises two main components: (i) a recognition element that recognizes/captures the target analyte and (ii) a transducer that converts the recognition event into a measurable signal. Electrochemical methods have been widely employed as the transducer mechanism because they can achieve higher analytical sensitivity and rapidity than techniques based on other mechanisms, such as optical or mass-based measurements. For point-of-use applications, the analytical system needs to be label-free. Although more convenient and less expensive, such label-free systems tend to sacrifice sensitivity and selectivity. To overcome this issue in label-free systems, the use of recognition elements with a strong affinity and high specificity to the detection targets, such as aptamers, has been proposed.

Aptamers are synthetic nucleic acids that fold into unique three-dimensional (3D) conformations capable of binding to a specific target, and have shown advantages over other molecules (such as antibodies) as recognition elements for biosensing. The advantages of aptamers include high affinity, specificity, thermostability, reusability (by reversible denaturation), ease of production, and modification with lower cost. Quite a few studies have reported the development of aptasensors using electrochemistry as a means of transduction, and a gold electrode modified with aptamers has been used for most of the electrochemical aptasensors reported. Several label-free electrochemical aptasensors using ferri/ferrocyanide (Fe(CN)₆³⁻/⁴⁻), a reversible redox couple, have been reported (e.g., a thrombin aptasensor using a gold electrode modified with aptamer).

Although many researchers have developed and reported on electrochemical aptasensors using redox markers for various analytes, electrochemical conditions of the aptasensors such as the electrode material or redox marker varied from one study to another. To our knowledge, there has been no study comprehensively comparing electrochemical conditions of aptasensors for practical use by researchers. One of the practical applications of aptasensor technology is the detection of norovirus, which is the major cause of nonbacterial acute gastroenteritis worldwide. In the present study, we aimed to improve electrochemical conditions for...
the reaction of the Fe(CN)₆³⁻/⁴⁻ redox marker toward the goal of application in the development of a norovirus aptasensor. Using the aptamer specific to murine norovirus (AG3), we explored electrochemical measurement conditions that provide high peak-current intensity in square-wave voltammetry (SWV), which is an analytical technique used for virus detection in electrochemical aptasensors.⁹⁻¹²

2. Experimental

2.1 Virus and cells

The prototype murine norovirus (MNV) strain, MNV-1 (American Type Culture Collection; ATCC, PTA-5935) was propagated on RAW 264.7 (ATCC; TIB-71) cell line monolayers with Dulbecco’s modified Eagle medium containing 10% fetal bovine serum. The propagated MNV particles were purified with gel chromatography (Illustra MicroSpin S-300 HR Columns, GE Healthcare Life Sciences), and the MNV titer was determined by plaque assay as described elsewhere.¹³

2.2 Enzyme-linked aptamer sorbent assay (ELASA)

Binding of aptamer AG3 to MNV particles was investigated according to a previously reported protocol.¹⁵ Briefly, the wells containing MNV of 1, 10, 100, or 1,000 plaque-forming unit (PFU) mL⁻¹ or PBS (negative control) were blocked with 200 μl of 5% skim milk in PBS-Tween 20 (0.05%, vol/vol; PBST) with a 10 nM mixture of unrelated PCR primers (Listeria monocytogenes primers hlyQF/R and L23SQF/R)¹⁶ overnight at 4°C with agitation (250 rpm). The plates were washed thrice with 200 μl of PBST and then incubated with 100 μl of 1 μM biotinylated AG3 aptamer for 1 h with agitation. Plates were washed with PBST and incubated at 100 μl/well with a 1-mg mL⁻¹ streptavidin-horseradish peroxidase (HRP) conjugate solution diluted 1:5,000 (vol/vol; Invitrogen, Carlsbad, CA) in PBS for 15 min with agitation. Residual conjugate solution was removed with three washing steps with PBST, and the plate signal was developed with the 3,3’,5,5’-tetramethylbenzidine (TMB) microwell peroxidase substrate system (KPL, Gaithersburg, MD) at 100 μl/well in accordance with the manufacturer’s instructions. The signal was allowed to develop for 6.5 min before reactions were stopped by the addition of TMB stop solution (KPL). The absorbance at 450 nm was then recorded with a microplate reader (Tecan Group Ltd., Männedorf, Switzerland).

2.3 Immobilization of DNA aptamer on working electrode

A gold nanoparticles screen-printed carbon electrode (GNP-SPCE), a screen-printed carbon electrode (SPCE), and a screen-printed gold electrode (SPGE) (DropSens, Asturias, Spain) were used as the working electrode, each with a projected surface area of approximately 12.5 mm² (circular shape with a diameter of approximately 4 mm). Immobilization of thiolated aptamer on the working electrode was carried out according to a previously described protocol,¹³ with slight modification. It was expected that the thiolated aptamer would be immobilized on GNP-SPCE and SPGE through the affinity between thiol and gold, but not on SPCE because of the absence of gold. Prior to immobilization, impurities on the electrode surface were removed by repeatedly oxidizing and reducing the electrodes in 25 mM (M = mol dm⁻³) phosphate buffer solution (pH 7.2) containing 1 M NaCl.¹⁷ In this electrode-cleaning process, the electrode potential was swept forward and backward between −0.5 and 0.7 V (vs. Ag/AgCl) 10 times at a scan rate of 20 mV s⁻¹ while being purged with nitrogen gas.

DNA aptamer was immobilized on the electrode by drop-casting 500 nM HPLC-purified murine norovirus-specific aptamer (AG3, aptamer nucleotide sequence described by Giamberardino et al.)² modified at the 5’ position with a 6-hydroxyxethyl disulfide group (HS-5’-AG3; Integrated DNA Technologies, USA) in a 20 mM Tris-ClO₄ buffer (pH 8.6) before incubating overnight at 4°C. Then the electrode was soaked in the ethanol solution of 1 mM β-mercaptoethanol (BME) for 5 min to back-fill empty regions of the electrode surface.¹² The BME back-fill was employed to reduce the non-specific adsorption onto the gold working electrode surface.

2.4 Electrochemical measurements

Electrochemical measurements were performed in single-chamber and three-electrode reactors. The working electrode was GNP-SPCE, SPCE, or SPGE. The counter and reference electrodes were Pt and Ag/AgCl in 1 M KCl, respectively. All measurements were performed at room temperature in a Faraday cage. Square-wave voltammetry (SWV) was performed using a bench-top electrochemical workstation (model 760E, CH Instruments, TX, USA) and the data were analyzed with ALS/CHI software version 16.03I (Tokyo, Japan). The SWV measurements were performed under the quiescent condition by sweeping electrode potential in negative direction from 0.32 to −0.48 V with a step potential of 4 mV, amplitude of 5 mV, and a frequency of 10 Hz. All electrochemical measurements were repeated at least four times for each sensor device or experimental condition.

3. Results and Discussion

3.1 Binding of MNV to aptamer AG3

In order to confirm the binding ability of aptamer AG3 to MNV particles, ELASA was performed for different immobilized MNV concentrations (1, 10, 100, and 1,000 PFU mL⁻¹). When 1 PFU mL⁻¹ of MNV was immobilized, the MNV/no MNV absorbance ratio was 0.97 ± 0.10, showing no observable binding of aptamer AG3 to MNV particles (Fig. 1). However, the MNV/no MNV absorbance ratios were substantially greater than 1 when 10–1,000 PFU mL⁻¹ of MNV was immobilized, and the absorbance ratio increased in an immobilized virus concentration-dependent manner (Fig. 1). This result confirms that aptamer AG3 captures MNV particles as reported in a previous study.¹²

3.2 Comparison of electrode materials

To compare the reactivity of electrode materials and the influence of the electrode modification process, we performed SWV for GNP-SPCE and SPGE with 4 mM potassium ferricyanide (K₃[Fe(CN)₆]) as redox probe. SPCE was also analyzed as a control without gold nanoparticles on the carbon electrode. GNP-SPCE had been used in previous studies,¹²,¹⁸,¹⁹ but a higher current is anticipated for SPGE.

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**Figure 1.** Binding analysis of aptamer AG3 and murine norovirus (MNV) with an enzyme-linked aptamer sorbent assay (ELASA). Different concentrations of MNV were immobilized on the plate. Ratios of the absorbance of wells with and without MNV are reported.
3.3 Effect of NaCl concentration on SWV peak current

We next examined the effect of NaCl concentration on the SWV peak current of 4 mM K₃[Fe(CN)₆] using SPGE modified with aptamer and BME. We used electrolytes with 0 to 3.0 M NaCl (Fig. 3a) and defined the SWV peak current relative to that at 0 M NaCl as “ΔCurrent”. The ΔCurrent value was highest at 1.0 M NaCl, and the value varied significantly with the electrolyte concentration (Fig. 3b), suggesting that electrolyte concentration is an important parameter to improve the sensitivity of aptasensors. Small peak intensities under low salt concentration were possibly caused by electrostatic repulsion. Negatively charged DNA aptamers inhibit the approach of Fe(CN)₆³⁻ to the electrode, but screening effect by high ionic strength can alleviate this electrostatic repulsion. The decrease of SWV peak current along with NaCl concentration increase to more than 1 M is most likely caused by the increase of the viscosity of the electrolyte. As the high viscosity decreases the diffusion coefficient of Fe(CN)₆³⁻, the square root of which is proportional to the SWV peak intensity, the higher NaCl concentration can suppress the SWV peak intensity, though this effect seems relevant only in the high NaCl concentration range. However, given that square root of the inverse of viscosity decreased by 10% if the NaCl concentration increased from 1 M to 3 M while the SWV peak intensity decreased by 20%, other effects that suppress the SWV peak intensity can exist. In addition to peak intensity, peak position also changed to the positive direction along with the increase of the NaCl concentration, which was caused by the rise in ionic strength of the electrolyte. Previous studies have not reported on the effect of electrolyte concentration on the performance of electrochemical aptasensors, but our results indicate that the electrolyte concentration should be taken into account when optimizing the electrochemical measurement conditions.

3.4 Investigation of the effect of [Ru(NH₃)₆]Cl₃

We investigated whether the electrochemical redox signal of K₃[Fe(CN)₆] could be enhanced by using hexaammineruthenium(II) chloride ([Ru(NH₃)₆]Cl₃), which is a well-known enhancer for the Fe(CN)₆³⁻/⁴⁻-based electrochemical sensors. Figure 4 shows the SWV responses of K₃[Fe(CN)₆] in the presence of 0.05 to 3.0 M NaCl with and without [Ru(NH₃)₆]Cl₃. The SWV peak current increased significantly with the addition of [Ru(NH₃)₆]Cl₃, indicating that the redox signal of Fe(CN)₆³⁻/⁴⁻ could be enhanced by using [Ru(NH₃)₆]Cl₃ as an enhancer. This result suggests that [Ru(NH₃)₆]Cl₃ could be a promising enhancer for the development of highly sensitive electrochemical aptasensors.
nium(III) chloride ([Ru(NH₃)₆]Cl₃). Because Ru(NH₃)₆³⁺ is a positively charged electroactive species, Ru(NH₃)₆³⁺ nonspecifically binds to negatively charged aptamer DNA backbone,²⁸,²⁹ accepts electrons from the electrode, and enhances the reduction of the redox probe (i.e., Fe(CN)₆³⁻) as an electron donor.³⁰ This reporter system has been adapted to norovirus aptasensors.¹²,¹³ The SWV measurements were performed with the bare SPGE in 25 mM phosphate buffer solution (pH 7.2) with 1 M NaCl, 4 mM K₃[Fe(CN)₆], and 0 to 35 µM [Ru(NH₃)₆]Cl₃. Three replicates of SWV spectra for SPGE are shown in Fig. 4, where the peak current varied among the replicas and there was no substantial effect of the concentration of [Ru(NH₃)₆]Cl₃ on the electrochemical redox signal of K₃[Fe(CN)₆]. Although 10 µM [Ru(NH₃)₆]Cl₃ was used in the previous studies,¹²,¹³ our result demonstrated that comparable electrochemical redox signal could be obtained even in the absence of [Ru(NH₃)₆]Cl₃ (Fig. 4). It was suggested that the use of [Ru(NH₃)₆]Cl₃, which is an expensive reagent, could be circumvented without losing the electrochemical sensing performance.

3.5 MNV titer-dependent response of the electrochemical aptasensor

In order to demonstrate the ability of the developed electrochemical aptasensor for target detection, we prepared a series of ten-fold dilutions of MNV (0 to 10⁵ plaque-forming unit [PFU] mL⁻¹) and performed SWV for SPGE in phosphate buffer with 1 M NaCl and 4 mM K₃[Fe(CN)₆] after applying 100 µL of the diluted MNV sample on SPGE followed by 30-min incubation. Separate SWV measurements with the electrochemical aptasensor (N = 3) were performed for each MNV titer. The representative square-wave voltammograms are shown in Fig. 5a. Well-defined and legible peaks were obtained, and the magnitude of peak current decreased in an MNV titer-dependent manner. As shown in Fig. 5b, the magnitude of peak current relative to a baseline decreased linearly with increasing titer of MNV over four orders of magnitude. This result demonstrated good linearity of the sensor response against different titers of MNV, indicating promising capability of the developed electrochemical aptasensor for MNV detection. The performance of our sensor for target detection was comparable to that of a previously reported aptasensor for MNV.¹³

4. Conclusion

Electrochemical aptasensors have been developed for various analytes, but the reported electrochemical measurement conditions for aptasensors vary among studies, and there has been no study comparing electrochemical conditions comprehensively for practical use. In the present study, we attempted to improve electrochemical
conditions for the redox reaction of Fe(CN)$_6^{3-}/4-$, toward application in aptasensors. MNV was used as a model virus for the development of an electrochemical aptasensor for norovirus detection. We firstly confirmed the binding ability of aptamer AG3 to MNV particles using ELASA. Subsequently, we compared working electrode materials and NaCl and [Ru(NH$_3$)$_6$]Cl$_3$ concentrations in terms of the SWV peak current. Our results demonstrate that an improved electrochemical measurement could be designed by using a SPGE as a working electrode, an electrolyte with 1 M NaCl, and 4 mM K$_3$[Fe(CN)$_6$] as a redox species. The electrochemical aptasensor exhibited a clear SWV response against di titer of MNV, demonstrating its potential for norovirus detection as well as other analytes.

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