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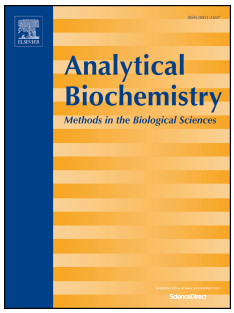

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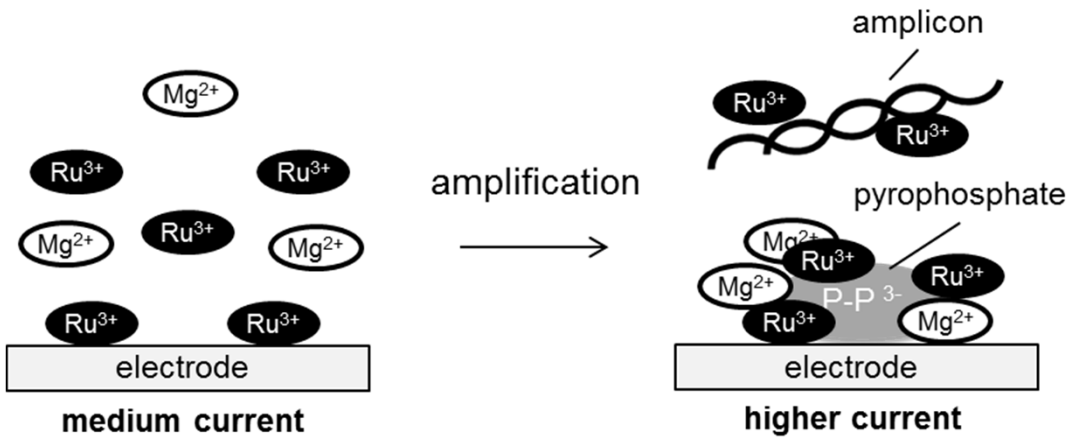
Graphical abstract



A novel voltammetric approach for real-time electrochemical detection of targeted nucleic acid sequences using LAMP

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Abstract

We have developed a novel voltammetric DNA chip for real-time electrochemical detection of targeted nucleic acid sequences using loop-mediated isothermal amplification (LAMP) and ruthenium hexaamine (RuHex) as the intercalative redox compound. A GspSSD DNA polymerase was used for LAMP owing to its tolerance of the intercalative redox compound. The electrochemical reaction of 1 mM RuHex in the LAMP solution was measured continuously by linear sweep voltammetry at 65°C using an electrochemical DNA chip. According to the LAMP reaction of the positive sample, the cathodic peak current of RuHex increased and the cathodic peak potential of RuHex shifted to negative voltage. The initial number of copies of the targeted nucleic acid was correlated with both the time when the cathodic current began to increase and the time when the cathodic potential began to shift rapidly. 10^3 to 10^6 copies/50 μL of the targeted nucleic acid were detected quantitatively and the detection limit was 10^1 copies within an hour. We expect these results to lead to the realization of simple and stable electrochemical real-time monitoring of targeted nucleic acids while also facilitating the implementation of electrochemical DNA chips in molecular testing.

Keywords:
Biosensor, DNA chip, LAMP, gold electrode, voltammetry, electrochemistry

1. Introduction

Molecular testing is now widely used in pathogen detection, DNA genotyping and RNA expression analysis. The enzymatic amplification of nucleic acid sequences, such as the real-time polymerase chain reaction (PCR) [1,2] and loop-mediated isothermal amplification (LAMP) [3-5], have become popular methods for quantifying targeted DNA or RNA molecules. However, current real-time PCR analysis requires specialized fluorochrome and an expensive optical monitoring system. Although it is possible for LAMP to monitor the reaction without fluorochrome labeling, it can only detect a gene in a single reaction.

Electrochemical methods for nucleic acid detection have been attracting attention as possible means of overcoming the drawbacks of the existing methods based on optical detection. They are expected to realize a portable, low-cost and multiplex testing. In the mid-1990s, electrochemical nucleic acid detection methods were based on the hybridization reaction between the probe-immobilized electrode and the targeted nucleic acid [6]. The specific nucleic acid after hybrid formation was detected by voltammetric measurement of the redox compound such as Hoechst 33258 [7,8]. However, complicated probe immobilization was necessary and the dynamic concentration range was narrow. In recent years, real-time electrochemical nucleic acid detection was achieved using an intercalative redox compound without the probe immobilization [9,10]. The first report was based on PCR using osmium compound (Os) as a redox probe [11]. The peak current of Os decreased with the amplification because of the slow diffusion of the Os-amplicon complex to the electrode surface. Since then, various papers have been published on real-time electrochemical monitoring of nucleic acid amplification such as the combination of helicase–dependent amplification and Os [12], the combination of LAMP and methylene blue [13,14], the combination of LAMP and crystal violet [15,16], the combination of LAMP and ruthenium hexaamine (RuHex) [17-19] and the combination of a rolling circle amplification and RuHex [20]. The common experimental condition reported by these papers was the use of redox compounds at low concentration (0.5 to 50 μM), because these compounds inhibit enzymatic amplification at higher concentration. However, compounds in the micromolar range were rather unstable with respect to the amplification condition and their binding properties to amplicon (double-stranded DNA) were variable at lower concentration. Consequently, many of the electrochemical methods exhibited significant drift of background signal. Furthermore, a number of papers reported the use of the square wave voltammetry (SWV) [11-14, 17-20], which is a highly sensitive electrochemical measurement technique. Although SWV is suitable for detecting low concentration of redox compound, it needs a special electrical circuit for detection and measuring speed is limited (typically several V/s). Therefore, it was considered that a more stable and simpler method was required to realize widespread application of molecular testing using electrochemical nucleic acid detection.

We have explored DNA polymerase tolerant for high concentration of intercalative redox compound and found that GspSSD DNA polymerase with a strand displacement activity is suitable for electrochemical monitoring of isothermal enzymatic amplification. In this paper, we present simple and stable real-time electrochemical detection of targeted nucleic acids.

2. Materials and Methods

2.1. Materials

A plasmid DNA (4 kbp) having parvovirus VP2 gene was used as a model for verification of a novel nucleic acid sequence detection method. The sequences of the LAMP primers for the model

plasmid were designated using Primer Explorer V 3.0 from Eiken Chemical Co. Ltd. The oligonucleotides were purchased from Life Technologies Japan. The primers for LAMP reaction were 5'-GAACATCATCTGGATCTGTACCAACCATCTCATACTGGAACTAGTGGC-3' (FIP), 5'-CTGTGCCAGTACACTTACTAAGAGTGTTAGTCTACATGGTTTACAATC-3' (BIP), 5'-GAGATATTATTTTCAATGGGATAGAAC-3' (F3), 5'-CAATGCTCTATTTGTTTGCCATG-3' (B3), and 5'-ACAGGTGATGAATTTGCTACAGG-3' (Lb). GspSSD DNA polymerase used for the LAMP reaction was obtained from OptiGene Ltd. Ruthenium hexaamine (RuHex) was purchased from Sigma-Aldrich. All other chemicals used were of analytical grade. The electrochemical DNA chip, consisting of 30 regions of gold electrodes (Φ=200 μm) patterned on a Pyrex glass substrate (W21 mm x D22 mm x H8 mm), silicone packing and a plastic cassette (Fig. S1), was purchased from Toshiba Hokuto Electronics, Co [21].

2.2. Methods

2.2.1. LAMP reaction condition

The LAMP reaction was carried out at 65°C by mixing 1.6 μM each of FIP and BIP primer, 0.12 μM each of F3 and B3 primer, 0.4 μM of Lb primer, 1.4 mM dNTPs, 20 mM Tris-HCl (pH 8.0), 60 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 0.1 % Tween20, 0.8 M betaine and 0.4 units/μL of the GspSSD DNA polymerase unless otherwise noted. 20 μL of the LAMP solution was used for turbidity measurement of the LAMP reaction using turbidity detection equipment LT-16 (Nippon Gene Co., Ltd). The amplification reaction was estimated by the time to threshold (Tth) of the turbidity. On the other hand, 50 μL of the LAMP solution containing RuHex was used for the electrochemical DNA chip. The concentration of amplicon was measured by QuantiFluor® ONE dsDNA System (Promega).

2.2.2. Electrochemical measurement

The electrochemical DNA chip was mounted on a customized holder using corning® LSE™ digital dry bath heater and heated at 65°C. The electrochemical signal of RuHex in the LAMP solution was measured by electrochemical analyzer ALS620e and multiplexer ALS684 (ALS Co., Ltd). The measurement conditions for square wave voltammetry (SWV) were: initial voltage = 0.15 V, final voltage = -0.3 V, increment = 0.002 V, amplitude = 0.05 V, frequency = 25 Hz. The measurement conditions for linear sweep voltammetry (LSV) were: initial voltage = 0.1 V, final voltage = -0.4 V, scan rate = 0.5 V/s. The cathodic reaction of RuHex was recorded for 60 min. The peak current and peak potential were determined by ALS 600 series software (version14.08J).

3. Results and discussion

3-1. Effect of RuHex concentration on LAMP reaction

We estimated the effect of RuHex concentration (in the range from 0.1 to 1 mM) on the LAMP reaction using GspSSD DNA polymerase. Figure S2 shows the results of real-time monitoring for turbidity of the LAMP reaction. The Tth was slightly increased with increase of RuHex concentration. However, the non-specific amplification was not observed and 10^2 copies of targeted nucleic acid were detected in all conditions. So we used 1 mM RuHex for LSV measurement, which is a basic electrochemical technique. Ahmed et al. [17] used 15 μM RuHex for real-time detection of LAMP because over 60 μM of RuHex inhibits the *Bst* DNA polymerase of LAMP reaction. The binding property of RuHex to double-stranded DNA is variable at such a low concentration range. Furthermore, micromolar range of RuHex is not easy to prepare stably and reproducibly. In our experiment, 1 mM RuHex hardly inhibits the LAMP reaction. It was considered that GspSSD DNA polymerase with strand displacement activity showed a higher resistance property than that of *Bst* DNA polymerase. Higher concentration of RuHex is useful for enhancing the stability of the electrochemical measurement.

3-2. Real-time electrochemical measurement of RuHex by LSV

We carried out the real-time monitoring of LAMP using 1 mM of RuHex by LSV. The positive sample contained 10^5 copies of targeted nucleic acid and the negative control was run without DNA. The baseline of the cathodic peak current (Ipc) and the cathodic peak potential (Epc) slightly drifted until the end of the measurement in negative control (Fig. 1A). On the other hand, Ipc suddenly increased (Fig. 1B and 1C) and Epc shifted to negative potential (Fig. 1D) from 30 min onward when a positive sample was used. Furthermore the Ipc and Epc were almost same as the negative control when the negative sample containing an excess amount of λ-DNA (10^9copies) was used (S6). The increasing of Ipc and shifting of Epc were considered to be associated with the specific LAMP reaction. The result of the Ipc increasing in a positive sample differed from those reported in previous papers [21,22]. To clarify this phenomenon, we estimated the real-time monitoring of LAMP using low concentration (25 μM) of RuHex by SWV. Figure S3 shows the time-course of the LAMP amplification curve for both the positive sample and the negative control. The baseline of the cathodic peak current (Ipc) gradually drifted in negative control. On the other hand, Ipc suddenly increased from 25 min onward in the positive sample, which is the same as in the case of LSV measurement. Then, we estimated the effect of magnesium concentration (2.0, 3.5, 4.0 and 4.5 mM) on the LAMP reaction. Figure 2 shows that the current increase was observed when magnesium concentration was over 4 mM. The turbidity of LAMP caused by the precipitation of pyrophosphate also increased when magnesium concentration was over 4 mM (data not shown). While it was confirmed that the targeted nucleic acid was amplified at least when magnesium concentration was over 3.5 mM, as determined by electrophoresis and concentration measurement of amplicon. From

these results, we speculated that the current increase is not caused by the formation of amplicon but also the precipitation of pyrophosphate. Figure 3 shows the schematic diagram of the real-time electrochemical LAMP using RuHex. In the negative sample, free RuHex in LAMP solution diffuses to electrode surface and generate the medium current by LSV (Fig. 3. Aa and Ab). In the positive sample with low magnesium, free RuHex is decreased due to its binding to amplicon so lower current is generated by LSV (Fig. 3. Ba, reported by previous papers). We confirmed that Ipc slightly decreased from 30min onward when magnesium concentration was 3.5 mM (Fig. 2). On the other hands, RuHex is co-precipitated with coproduced pyrophosphate on the electrode surface in the positive sample with high magnesium concentration. Then higher current is generated by LSV (Fig. 3. Bb, reported in this paper). The Ipc behavior in real-time monitoring of RuHex is caused by co-precipitation of RuHex and negatively charged pyrophosphate produced as LAMP coproduct on the electrode surface. The experimental conditions, such as species of polymerase, materials of electrode, magnesium concentration and measurement technique (SWV or LSV), may affect the formation of the pyrophosphate. Almost all the papers on real-time electrochemical monitoring amplification report the use of SWV [11-14, 17-20], which is a highly sensitive electrochemical measurement technique. However, it needs a special electrical circuit for detection and the measuring speed is limited (typically several V/s). We could carry out the electrochemical LAMP reaction by LSV, which is a basic electrochemical technique capable of 1000-fold faster scanning (over 10^4 V/s). This result shows a possibility of fast and low-cost electrochemical instrument for real-time nucleic acid detection.

3-3. Optimization of measurement conditions

Figure S4 shows the effect of scan rate (0.1 V/s, 0.5 V/s and 1.0 V/s) on RuHex measurements by LSV at 10^5 copies of targeted nucleic acid in 50 μL of LAMP solution. The initial Ipc increased with increase of scan rates. The initial Ipc was correlated with square root of scan rate (r^2=0.99). The current increasing times at each scan rate were 28, 25 and 27 min, respectively. The least variability was obtained at 0.5 V/s. From these results, we used Ipc measurement of RuHex at 0.5 V/s for real-time detection.

When the turbidity of the LAMP reaction was measured using a transparency tube, Tth was around 15 and 21 min at 10^5 and 10^3 copies of targeted nucleic acid, respectively. The current increasing time was about 10 min later than Tth of turbidity measurement. The DNA chip has a larger solid-liquid interface than the transparency tube. Furthermore, the tube is made of low-adsorption material. It was supposed that the active substances of the LAMP solution, such as enzyme and primers, decreased due to adsorption on the chip surface. Therefore, we optimized the composition of the LAMP reaction for the DNA chip detection. Figure S5 shows the effect of enzyme (x 1 and x 2) and primer (x 1, x 1.5 and x 2) concentration on the current increasing time

using the electrochemical DNA chip. When a twofold enzyme was used for the LAMP reaction (40 units), the current increasing time improved to 22 min at 10^3 copies. On the other hand, addition of twofold primers had no effect on the current increasing time.

3-4. Real-time quantitative detection

Different concentrations of targeted nucleic acid (10^1, 10^2, 10^3, 10^4, 10^5, 10^6 copies/50 μL) were amplified at 65°C and the cathodic current of RuHex was measured simultaneously up to 60 min using LSV for quantitative analysis. The twelve electrodes on the DNA chip were used for data analysis. Figure 4 showed semi-logarithmic calibration plots of the current increasing time as a function of initial copy number. The calibration plots were roughly linear when the number of copies of the targeted nucleic acid was higher than 10^3. The linear relationship between 10^3 and 10^6 copies revealed the square of the correlation coefficient 0.93 and the slope -2.21. The standard deviation (SD) of each point was 1.0, 0.4, 0.4 and 0.0 min, respectively. From these results, the novel electrochemical method delivered reproducible and quantifiable measurement. On the other hand, the plots deviated from linearity as a function of amplification time at a lower sample concentration (< 10^2 copies). The SD at 10^2 copies was 10.0 min and the four electrodes were only positive and eight electrodes were negative at 10^1 copies. In the isothermal amplification reaction, it was speculated that the motion of the targeted nucleic acid or amplicon was dominated by the diffusion. The diffusion distance of targeted nucleic acid in the LAMP solution for 60 min was calculated to be about several hundred micrometers by references to both the diffusion coefficient of the DNA fragment in water [23] and the viscosity of betaine [24]. We used the electrochemical DNA chip having 30 regions of electrodes on the plane surface for 50 μL of the LAMP solution. The gap between electrodes was 2 mm. Considering the diffusion distance, the volume of the LAMP solution covered by an electrode was no more than 1.7 μL (50 μL/30 electrodes), which contains only a few molecules of targeted nucleic acid at 10^2 copies/50 μL. The instability of detection below 10^2 copies/50 μL of targeted nucleic acid could be explained in terms of the structure of the DNA chip used in the experiments. We achieved over 3 decades of dynamic concentration range with detection limits as low as 10^1 copies of targeted nucleic acid. The detection limit was almost same as real time detection based on the turbidimetry (Fig. S2).

4. Conclusion

We have developed a novel real-time electrochemical detection of targeted nucleic acid sequences. Using GspSSD DNA polymerase, a higher concentration of RuHex was adaptable to LAMP and highly sensitive and quantitative analysis were achieved within 1 hour by LSV. The good reproducibility was obtained between 10^3 and 10^6 copies of targeted nucleic acid and detection limit

was 10^1copies. We expect to apply this simple and stable method for detecting specific nucleic acids in medicine such as for the diagnosis of infectious diseases and in expression analysis of RNA.

Acknowledgements

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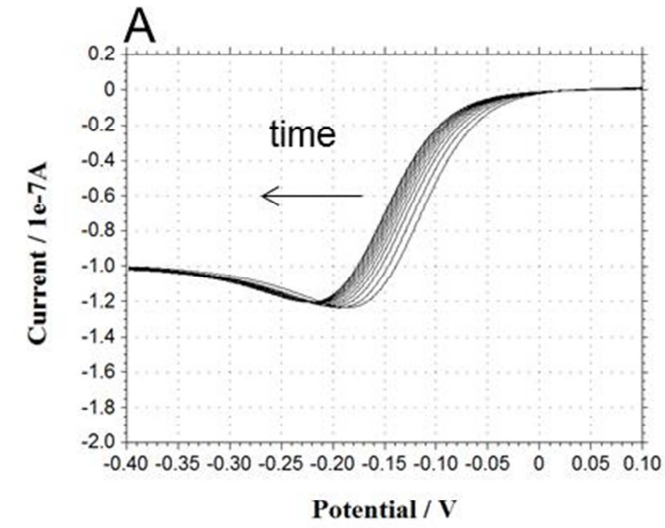
Figure captions

Fig. 1. Linear sweep voltammograms of 1 mM RuHex in 50 μL of LAMP solution at 65°C using (A) negative control and (B) 10^5 copies of positive sample. The scan rate was 0.1 V/s and the voltammograms were recorded for 60 min at every 5 min (right to left). (C) Ipc of RuHex vs amplification time and (D) Epc of RuHex vs amplification time.

Fig. 2. Effect of magnesium concentration on LAMP reaction. The concentration of the targeted nucleic acid was 10^5 copies/50 μL. LSV was measured at every 5 min.

Fig. 3. Schematic diagram of the real-time electrochemical LAMP using RuHex in (A) negative sample and (B) positive sample. Magnesium concentration is (a) low and (b) high.

Fig. 4. Calibration plots of current increasing time vs logarithmic input of the targeted nucleic acid. The concentration of GspSSD polymerase was 40 units/50 μL. LSV measurement was recorded at every 1 min. The error bars indicate the standard deviation of signal derived from 12 electrodes on the DNA chip.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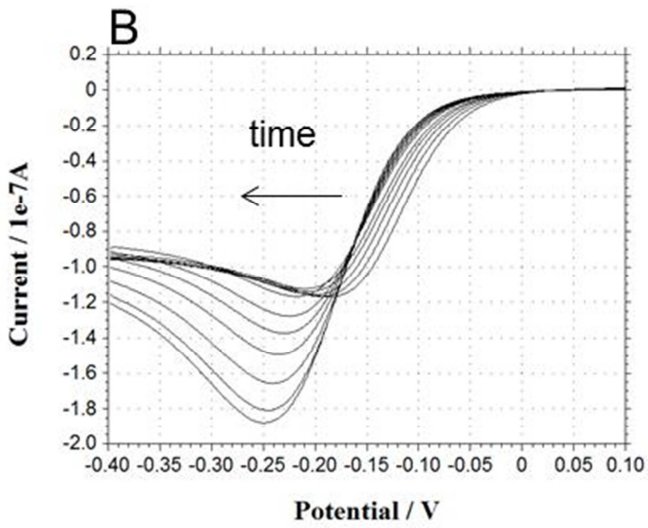
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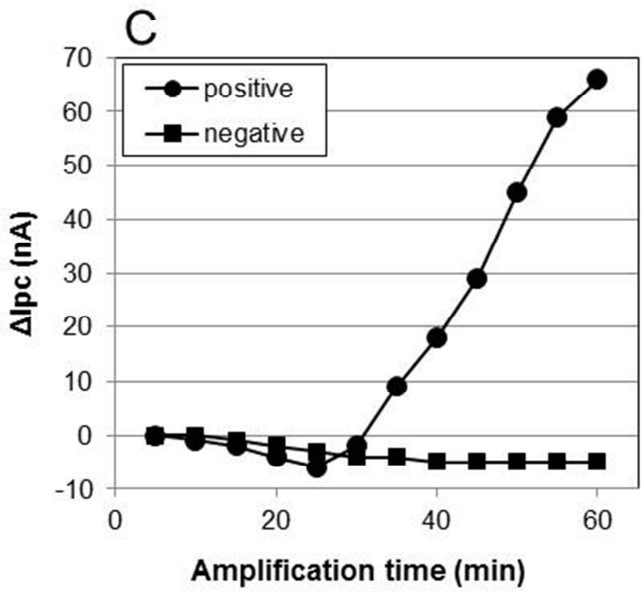
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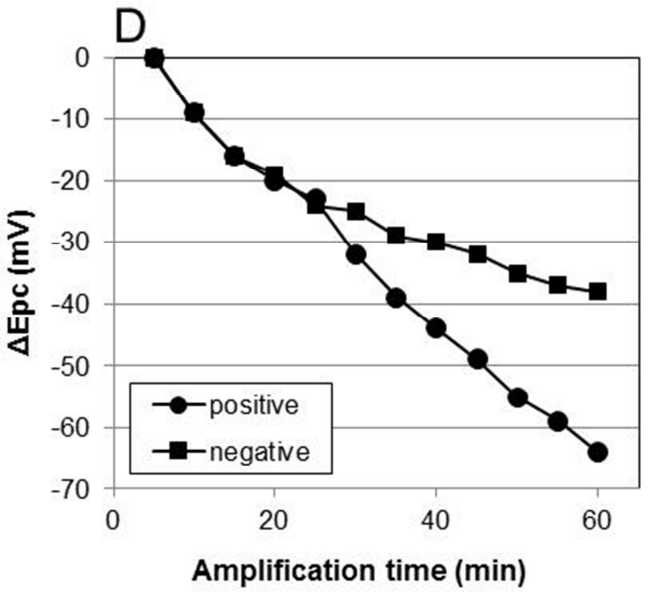
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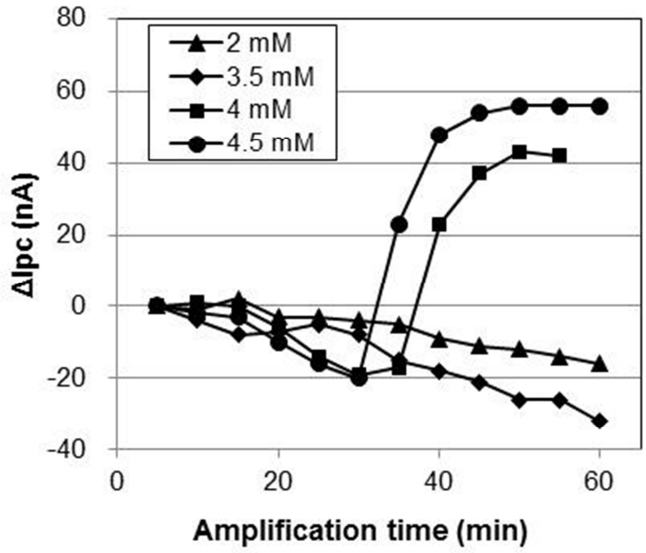

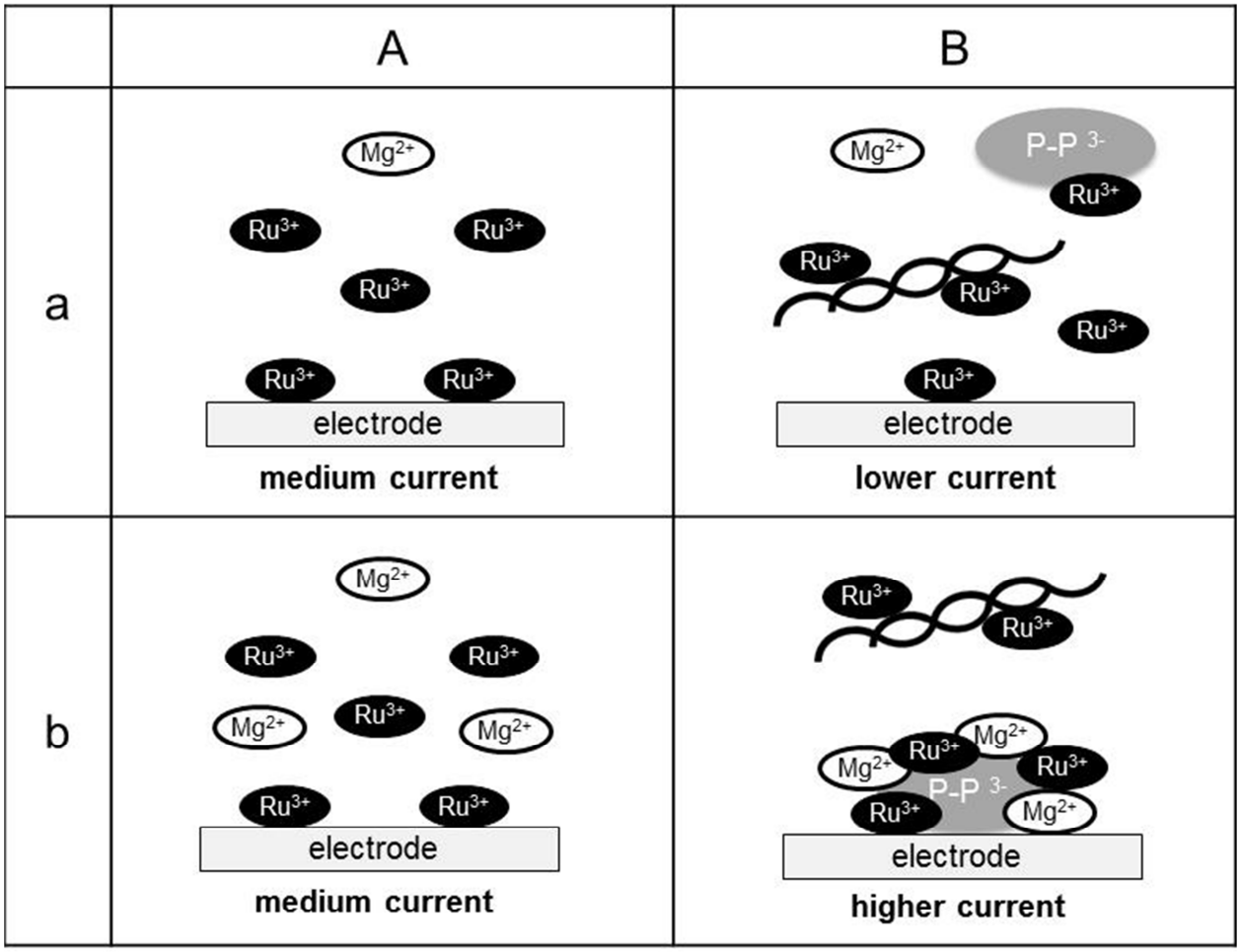
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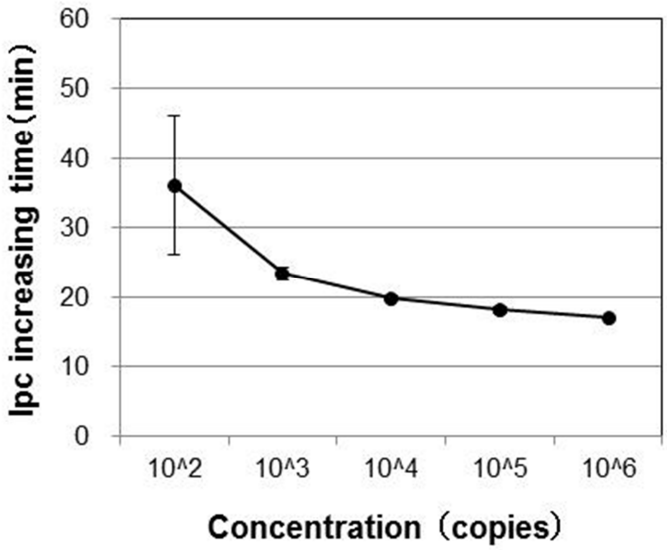

Highlights:

·Simple and stable real-time electrochemical detection of nucleic acid sequence.
·Novel voltammetric DNA chip monitoring of enzymatic amplification was achieved.
·Detection limit down to 10^1 copies within an hour.