Accumulation of amplified target DNAs using thiol/ biotin labeling, S1 nuclease, and ferrocene–streptavidin–magnetic system and a direct detection of specific DNA signals with screen printed gold electrode

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Abstract

Combinations of PCR-based amplification platform using 5' thiolated and biotinylated specific primers, S1 nuclease–PCR products treatment, ferrocene–streptavidin (Fc–Stv)–magnetic binding for DNA accumulation, and screen printed gold electrode for the DNA allocation, were applied to Hoechst 33258-induced DNA aggregation and signals induction system for direct signals detection and DNA quantification in food samples. Thiolated and biotinylated at each 5' terminus enabled DNA purification through S1 nuclease treatment for primers and non-specific DNA elimination and enabled DNA trapping with a ferrocene–streptavidin–magnetic system. This facilitated the accumulation of target DNAs at higher concentration, resulting in enhanced signals. After allocation of DNA on the surface of gold electrode via thiol binding, intensity of DNA signals through these treatments could be measured directly after being induced by Hoechst 33258. Wider amplitude changes in anodic current peaks between negative and positive samples (increasing from 3.70 to 10.10 \(\mu A\)) compared with those applied with no treatment combinations (decreasing from 3.92 to 1.23 \(\mu A\)) were observed. This enhancement of the signals allowed a greater efficiency of DNA quantification. When this combination was used for GMOs content estimation in reference samples, results revealed an improved accuracy from 66% to 96%. The combined biosensor system, although more costly than the standard Hoechst 33258/carbon electrode system, provided an alternative choice for DNA quantification, offering labor-free immobilization of probe onto electrode surface, easy test administration, and efficient semi-quantitative test without expensive instruments.

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1. Introduction

Classical DNA analysis for food testing requires steps of DNA extraction from food matrices, specific target DNA amplification, and detection of the obtained target DNAs. Result analysis based on this approach depends on further steps of DNA fragment size determination via either gel visualization, restriction endonuclease, banding profile, or nucleotide sequence, all of which are time and labor consuming and provide only limited degree of qualitative data \cite{1,2}. In several cases, for which semi-quantitative data especially on threshold amount of DNA in food samples need to be clarified, an application of real-time quantitative amplification was carried out despite its high cost, dependence on expensive instruments, and complicate analysis. This can be conducted at few major laboratories. In addition, when the procedures rely on instruments, the application of DNA in food testing was then limited to an extent that no point of care test could be conducted.

Recently, a biosensor system using electrochemical techniques has been demonstrated as an alternative choice for nucleic acid-based diagnosis because of economic and technical advantages \cite{3}. The system needs both biomolecules with highly specific recognition elements for
target nucleic acid and a transducer that converts molecular recognition events into quantifiable signals [4]. When this system was applied to food analysis, whose post-process nucleic acid contents were miniscule, a successful detection depended on both the amplification of target signals and the organization of specific recognitions for measurement of signals in a very specific manner. Traces of target nucleic acids can be amplified via a widely accepted technique of DNA amplification namely PCR [5]; however, organizing the recognition signals in a specific way for direct measurement was another key factor for the successful determination.

In a previous report, our group had developed a method based on an electrochemical biosensor to help determine semi-quantitative amount of DNAs. The method required no probe and the immobilization step for the organization of recognition signals [6], thus, reducing time in pre-immobilization processes. The technique, based on highly specific target DNA amplification system using PCR and the aggregation phenomenon of target DNA in the presence of Hoechst 33258, demonstrated how the principle of electrochemical biosensor could be applied for the semi-quantitative analysis of target DNAs in samples. This allows a cost-effective and rapid determination complying with available enforcing regulations. Determination based on this system was very satisfactory for feedstuff analysis [7].

Although the method has several merits, its detection mechanisms, based on the remaining charges on the electrode that bind with Hoechst 33258, were an indirect measurement. This indirect measurement may limit the efficiency of quantitative determination in terms of signal’s amplitude detection, as well as a possible false positive signal measurement generated after non-specific DNA amplification if the PCR system was not perfectly calibrated. Thus, when the determination was not based only on qualitative detection of target DNA, as in feeding stuffs, but also on threshold amount of DNA clarification and when specific primers were designated based on the accepted official PCR protocols [8] which might not be fit for sensor, a direct organizing of the recognition signals is crucial.

One of the approaches used to directly measure signals was based on enzyme labeling to generate the amplified signals via either a production of an electroactive compound, or bio-electrocatalytic redox transformations, or the biocatalyzed precipitation of an insoluble product at the electrode [9]. Although large signal accumulation from enzyme labels can be achieved, a labeling step by the enzyme is not straightforward, and has limited label instability.

Recently, several applications of chemical bindings of DNA molecule to target surface were demonstrated [10,11]. One of these was the biotin and ferrocene/streptavidin (Fc–Stv) binding, which provided strong affinity and extreme stability. An exploitation of this phenomenon had already been demonstrated for bio-electrocatalytic detection of viruses via the binding of DNA to a sensor surface [12,13]. Another example was an application of thiol/Au to allocate the binding of oligonucleotides on the Au surface [14]. The bindings of DNA via thiol group to the surface of the gold electrode had been applied to detect DNA deamination [15].

In this report, we have applied altogether the phenomenon of (1) thiol/Au to directly assist the allocation of DNA signals on the surface of the gold electrode, (2) an enzymatic reaction of exonuclease treatment to reduce the primers and non-specific target after PCR, and (3) the conjugation of biotin and Fc–Stv for signal DNA trapping and enriching before the induction of the signals using aggregation induction by Hoechst 33258. These applications provided the detection of specific DNA signals through the direct accumulation of electrons onto the electrode surface while eliminating labor-intensive steps of immobilization and DNA labeling on the surface of the electrode. In addition, non-target signals that might interfere with the detection precision were also eliminated. We also demonstrated the qualitative and quantitative detection of genetically modified (GM) maize, event MON810, in target specimens required for significant levels of a GM co-mingle test as a model and compared with the results from the original system without combined treatments [7].

2. Experimental procedures

Positive target DNAs and known copies of DNA were obtained from cloned DNA fragments corresponding to CrysA(b) portion of MON810 and the SSII b gene, a specific internal gene of maize. GM samples were test samples and reference samples from Food Research and Testing Laboratory, Faculty of Science, Chulalongkorn University, Thailand. Methods of DNA extraction and DNA detection were the Japanese Official Method as described [16,19]. A design of primers with thiol or biotin at 5′ terminus, was based on the sequence of CrysA(b) of MON810 and the common SSII b gene. A forward primer 5′SH-aaaaaagatgcctctcctctggttgacctgacag-3′ and a reverse primer 5′Biotin-aaaaagatgcctctcctctggttgacctgacag-3′, corresponding to 250 nucleotides of CrysA(b) gene, and 5′SH-aaaaaactcctatttggactctg-3′ and 5′-Biotin-aaaaaactcctatttggactctg-3′ corresponding to the SSII b gene were synthesized by Fasmac Co., Ltd. (Fasmac, Japan). Another set of primers with no labeling were also prepared and used for comparison. For the signal amplification, DNAs from both specimens, a positive DNA, and a negative DNA were amplified in 50 μL reactions using 2 μL of DNA, 1 U of Ampli Taq Gold (Applied Biosystem, USA) with 50 μM MgCl2 and 10 pmol of each primer. DNA amplification was carried out for 40 cycles for 1 min each at 94, 58, and 72 °C. PCR products obtained from the reaction with unlabeled primers were mixed with 50 μM Hoechst 33258 in PBS buffer volume by volume and detected for electrochemical signals using carbon screen
printed electrode as described [7]. For PCR products with labeled primers, the elimination of the remaining primers was carried out with 0.1 U/reaction of S1 nuclease (Promega, USA) with 10 min incubation at 37 °C and 10 min heating at 80 °C. The enrichment of the DNA signals was then carried out by Fc–Stv binding activities (Sigma, USA). Target DNAs were later trapped at the bottom of the tube by a magnetic device and re-dissolved to 25 μL volume. Binding of signals to the Au electrode surface took place in 20 mM PBS at 4 °C for 30 min with screen print gold (area: 2.64 mm²) and Ag/AgCl as a reference electrode. The detection of electrochemical signals of 25 μM Hoechst 33258 was further carried out in PBS buffer using linear sweep voltammetry (LSV) as described earlier [7]. All measurements were performed in triplicates at 25 °C. In the experiments, anodic current peaks of samples with the presence and the absence of a template, a biotinylated primer, and the trapping with Fc–Stv were also examined.

In order to quantify DNA, cloned portions of CryIA(b) of MON810 and SSIIb in pCR4™ (Invitrogen, USA) were used as known copies of reference. Two different positive samples, 10% and 0.9% GMOs (the latter based on EU regulation) were further employed. DNA was extracted as described [16]. Measurements were taken after 30 cycles of amplification of the reference genes to limit DNA saturation using similar conditions, with 100, 10, 1, 0.1, 0.01, 0.001, 0.0001, and 0.00001 ng of DNA templates and compared with those of the positive samples [7]. Calibrations of copy number of target genes were based on the relationships between amplified copy numbers of the CryIA(b) gene and the SSIIb gene using a known copy number of plasmids as the template and an anodic current peak drawn against the copy numbers via a standard curve method [17]. The content of MON810 constituents was determined based on the ratio of target CryIA(b) of the MON810 related cells per total cell numbers estimated from the copy number of the SSIIb gene with number of coefficient as described [16]. A comparative study on DNA quantification using Hoechst 33258/carbon screen printed electrode was also carried out [7].

3. Signal amplification, screening, and accumulation

The configuration of DNA detection system using labeled primers and S1 nuclease treatment comparing with the original model was depicted in Fig. 1. The system combined a PCR-based DNA amplification platform, specific to target CryIA(b) gene, using 5’ thiolated and biotinylated primers, respectively, and the S1 nuclease treatment to enhance specificity through elimination of the remaining primers and some non-specific products. Those biotinylated and thiolated at 5’end of both termini enabled the binding of DNA products with ferrocene–streptavidin and magnet, as well as the binding of DNAs to the electrode surface. After the bindings, a signal induction using Hoechst 33258 was imposed. These bindings were expected to enrich signals by increasing both DNA concentration and signal accumulation via free electrons associated with an absorbed DNA–Hoechst 33258 complex directly on the electrode surface. When this was compared with our previous model using normal primers [7], measured signals were decreased as a result of the loss of Hoechst 33258’s free electrons due to the slow diffusion DNA–Hoechst 33258 complex suspended in the sample solution over but not on the electrode surface.

In this study, the genetically modified maize, event MON810 was selected as a target specimen because of the

Fig. 1. Schematic representation of the sensing configuration: (A) using thiol and biotin primers for PCR amplification, S1 nuclease for elimination of remaining primers and Fc–Stv and magnetic trapping for signal enrichment before Hoechst 33258 induction and measurement on gold electrode; and (B) using original model with Hoechst 33258 induction and measurement on carbon electrode.
EU’s requirement for a rapid qualitative detection as well as a quantitative determination to the level below the defined 0.9% threshold baseline [8]. MON810 materials were confirmed at the beginning using standard PCR protocol [18]. The DNA had also been extracted and amplified using standard conditions described in Japanese Official Method [16,19]. In the reaction, both 5’s thiolated and non-thiolated primers in enzymatic PCR amplification, a treatment and a non-treatment of S1 nuclease enzyme to DNA products, and a Fc–Stv binding or non-binding were compared (Table 1). Anodic peaks among combination of treatments were found in the range of 3.70 (negative sample) to 10.10 mA (positive sample), respectively. This increasing of anodic peaks observed was due to the binding of DNA–Hoechst 33258 complex on gold electrode. The results were totally different from the system using carbon electrode on which decreasing of signal was addressed. High anodic peak value was also observed when Fc–Stv was successfully applied. This implied a merit of signals accumulation via biotin–Fc–Stv binding. The anodic peak value, among samples treated with S1 nuclease, were higher than those without treatment. This might be possible due to the remaining of primers could compete with the target DNA for electrode surface binding. In order to investigate the quality of DNA after treatments, gel electrophoresis of DNA were carried out. Results of visualized DNA products (arrow) were shown in Fig. 2. Based on official method, both target DNAs and non-specific products were detected. It was found that both the treatment with S1 nuclease enzyme and the accumulating DNA through the binding of Fc–Stv provided means to eliminate either unincorporated primers or some non-specific DNAs that were generated from the false mismatch of PCR processes out of the target element and accumulation of the signals. S1 nuclease had been known to induce degradation of some non-specific DNA hetero-duplex [20]. In the test, the nuclease activity was expected to act on the single stranded portion of DNA primers available in the last round or on portions of single stranded DNA generated from mismatches [21]. This was supported by evidence that no trace of small DNA molecules could be visually detected by gel electrophoresis compared with non-treated results. Although S1 nuclease had been commonly used in molecular detection, especially when single stranded DNA was involved, its application in an electrochemical

Table 1
Anodic current peaks measurement of the samples either with combined system or original model using Hoechst 33258

| With combination of treatments | Anodic current peak measurement (A), (SD) |
|-------------------------------|------------------------------------------|
| $5'$ SH                       | $5'$ biotin | S1 nuclease | Fe–Stv trapping |
| +                             | +          | +           | +              | 10.10, (0.08) |
| +                             | +          | −           | +              | 7.23, (0.09)  |
| +                             | +          | +           | −              | 5.72, (0.10)  |
| +                             | +          | +           | −              | 5.08, (0.08)  |
| +                             | −          | +           | −              | 4.95, (0.12)  |
| +                             | −          | −           | −              | 4.82, (0.08)  |
| −                             | +          | +           | +              | 3.54, (0.11)  |
| −                             | −          | −           | −              | 3.42, (0.10)  |
| −                             | −          | −           | −              | 3.70, (0.08)  |

Without combination of treatment**

| GMOs negative (MON810 –) sample | 3.92, (0.07) |
| GMOs positive (MON810 +) sample  | 2.18, (0.09) |

+ = treatment, − = non-treatment.
*ntc = non-template control.
**measurement using gold electrode.

Fig. 2. Quality of target CryIA(b) gene products, having both thiol and biotin at 5’ termini after treatment with S1 nuclease and Fe–Stv. Lane 1: control target DNA; Lane 2: non-template control; Lane 3: treated with S1 and Fe–Stv (eliminate & trap); Lane 4: treated with Fe–Stv (trapping only); Lane 5: treated with S1(eliminating only); Lane 6: non-treated; Lane M: 100 nucleotide ladder.
detected was relatively limited [22]. The treatment of S1 nuclease here was the first demonstration in primer elimination before the electrochemical detection. Similarly, the application of Fc–Stv had also been demonstrated extensively for the amplification of detection signals [23]; however, its use in the electrochemical detection was focused mainly on improving the sensitivity of surface-based DNA hybridization [24,25] rather than the accumulation of signals as being applied in this study. The described treatment of both S1 nuclease and Fc–Stv solved problems of specificity and improved signal intensities.

In a previous paper, signal measurement was indirectly carried out based on the binding of Hoechst 33258 molecules to the DNA products during last step, irrespective of target, or non-target DNAs, or saturated or unsaturated products. This might cause false positive signals due either to the trace of imperfect PCR or primer dimers. Although Hoechst 33258 was neither binding to single strand DNA nor primer nor short primer dimers [26], to our knowledge, non-specific DNA having length more than 50 nucleotides might response to Hoechst 33258, rendering fault signals. The greater of the DNA amount was available in the test environment, the more binding of Hoechst 33258 and induced DNA aggregation was expected. When this was applied to the electrode, DNA aggregation increased the binding of free electrons at DNA molecules but decreased free electrons at the electrode surface, which then resulted in decreased anodic current peaks measured [6]. Thus, using this system requires PCR system validation not to create non-specific signals. In contrast, thiolated primers provided DNA products that had Au-electrode binding affinity. Then after the S1 nuclease treatment and Fc–Stv induced accumulation of the signals, the DNA was then immobilized on the gold electrode, allowing signals to be directly measured. Our system still relied on Hoechst 33258, because its introduction helped bypass the need of DNA hybridization, therefore, avoiding the necessity of probe preparation and laborious hybridization steps, while still providing a valid platform in direct signal measurement.

4. Comparative studies on anodic current signals from direct and indirect measurements

Comparative studies on anodic signals based on direct measurement using gold electrode and indirect measurements as appeared in previous work using both screen printed carbon and gold electrode (area, 2.63 mm²) was described in Fig. 3 and Table 1.

Enzymatic reactions both by DNA polymerase and S1 nuclease, enhanced target signal amplification and reduced non-specific traces while allowing signals from PCR products, but not the remaining primers, to get direct access for electrochemical measurement. When non-thiolated primer was employed, anodic current values were at a low range, between ~1.23 (in the presence of DNAs) and 3.92 µA (in the absence of DNAs). The decrease in anodic peaks was in proportionate to the increase in the DNA amount of DNAs in the reactions similar to the previously observed study [6]. In contrast, thiolated primers exhibited an extra increase of the signals with broader amplitude of anodic peaks ranked between 4.80 (in the absence of DNAs) and 10.10 µA (in the presence of DNAs). Increasing in the amplitude of anodic peaks signals was due to the binding of DNA on surface of electrode.

In the experiment, anodic peaks of target signals after enriching DNAs (trapping of DNA products via Fc–Stv–magnetic and SH binding of DNAs) on the gold electrode were more than 5 times those of the non-binding system (10.10 µA:1.23 µA) (Table 1). DNA amplification and trapping with Fc–Stv allowed signals to be accumulated. In addition, the binding with thiol group allowed DNAs to bind directly on the gold surface at greater efficiency [27]. In the previous report [7], the PCR system for semi-quantification of DNA from bovine species using carbon screen printed electrode did not render a wider amplitude of anodic current peak (0.90–2.30 µA) [7]. It demonstrated results with approximately 87% accuracy. The wider span of anodic current peaks from 3.71 to 10.10 µA in direct system, comparing with 1.23–3.92 µA of indirect one on the same gold electrode this time, allowing copy number to be calibrated at more accuracy and in turn enable the technique to be better applied for a quantitative analysis, especially when the semi-quantitative result was required in GMOs regulation.

In the experiments, anodic current peaks observed in Fc–Stv and thiol DNAs with S1 nuclease treatment on surface of the gold electrode were higher than those in the non S1 nuclease treatment. The current peak was also lower in treatment containing no biotin (leading to products having no ability to accumulate by Fc–Stv) (Table 1). Changes in anodic current peaks in both cases were direct evidence of S1 nuclease and biotin–Fc–Stv functions on signals accumulation. If S1 nuclease was not treated, leftover primers could occupy the DNA binding area on the electrode resulting in bad noise to data ratio of the signals. S1 nuclease treatment helped eliminate the leftover primers and some mismatch DNAs, thus, decreased any chance for non-specific surface binding competition. Biotin groups, on the other hand, facilitated the accumulation of the signals via Fc–Stv–magnetic trapping [23,25,28]. In the test, a lower anodic current peak was observed in non-biotin products despite the full capacity of thiol binding. This suggested a key role of biotin–Fc–Stv coupled with a magnetic field in signal accumulation. DNA products without a biotin portion at their end termini will then never deliver greater signals than those with biotin.

In our experiment, the thiol group also played an important role in the binding of signals on the surface of the gold electrode. The binding mechanisms of the thiol group to gold electrode had been addressed elsewhere [29], and the application of this technique in electrochemical biosensor was demonstrated extensively [30–32]. However,
there had been no report on the application of thiol-Au binding on the gold electrode together with redox signal induction using Hoechst 33258 as DNA aggregator on electrode surface. This experiment demonstrated for the first time the combined effects of thiol, biotin, S1 nuclease, Fc–Stv–magnetic binding, a gold electrode, and Hoechst 33258 in enhancing signals and measuring direct signals. Signal enhancement through this system will help answer the need to quantitatively determine target DNA for semi-quantitative studies.

5. Quantitative analysis of signals for GMOs content determination

With a broader signal detection amplitude using thiol, biotin, S1 nuclease, Fc–Stv–magnetic field, and a gold electrode, semi quantitative analysis to determined the amount of GMOs (event MON810) in target samples can be performed. Calculation of the DNA copy number was based on the direct measurement of anodic peak signal transduction, induced by the aggregation of DNA molecules that were amplified from reference plasmids ranging from $10^6$ to $10^7$ copies. Instead of using the kinetic analysis on fluorescent signals as monitored by real-time PCR [16], a relationship between anodic current peaks and DNA concentration was generated (Fig. 4). The copy number of each sample based on CryIA(b) and SSIIb genes could be obtained. The amplification of CryIA(b) and SSIIb genes was fixed at 30 cycles to detect signal before PCR products reached saturation. The representative relationship between CryIA(b) gene concentration (copy number/50 µL reaction) in log and anodic peak (Y; µA) was also shown, with $Y_{\text{CryIA(b)}} = 1.0988X + 2.8525$ with $R^2 = 0.9966$ where $Y$ was anodic peak from each sample, and $X$ was gene concentration. Similarly, the relationship between SSIIb copy number and DNA concentration was $Y_{\text{SSIIb}} = 1.102X + 2.8292$ with $R^2 = 0.9951$ (not shown). Anodic measurements for both genes and their corresponding copy numbers (anti log) were described in Table 2. The relationships were used to estimate copy number of both genes in reference samples, 10% and 0.9%. In the experiment, measurement of GMO content using previous method with carbon electrode provided the relationship of these gene and their copy number as $Y_{\text{CryIA(b)} \text{carbon}} = -0.1849X + 2.2069$ with $R^2 = 0.9991$ and $Y_{\text{SSIIb carbon}} = -0.1845X + 2.0358$ with $R^2 = 0.9959$ (not shown). The calculation of GMO content (%) was based on ratios of copy numbers of recombinant DNAs (CryIA(b)) and copy numbers of endogenous SSIIb sequences in each sample (to formulate total cell numbers) using equation GMO% = copies of CryIA(b)X100/(copies of SSIIbX0.38) where 0.38 was number of coefficient (detail method of calculation see [17]). When the calibration was approaching at low
GMO content (0.9%), estimation value through carbon electrode provided a result with lower accuracy (66.80%). In contrast, estimation value obtained from the measurement using combined system on the gold electrode was higher (96% compared with 66.80%).

From the estimation, the GMO contents of 10% GMO and 0.90% GMO samples were 9.436% and 0.865%, respectively, a 94.36% and 96.11% close to the reference value. An increasing in anodic current amplitude provided an opportunity for calibrating at greater efficiency. The results provided an example of how to enhance signals for quantitative detection. By using the 5'-labeling of thiol and biotin, S1 nuclease treatment and binding of target DNAs on gold electrode, the determination of GMO contents could be determined. This provided an alternative choice for quantitative analysis in addition to real-time PCR or competitive quantitative PCR. The threshold amount of 0.90% GMO contamination in the experiment can also be estimated from the anodic current peak value, 5.30 μA.

Such reference can be routinely used if only a rough content was expected on the genuine seed detection using the same reference plasmid.

The measurement using a fabricated sensor based on thiol, biotin, S1 nuclease, Fc–Stv–magnetic binding, Hoechst 33258, and a gold electrode in this experiment requires no additional modification, as found in immobilization, in the steps of signal induction. Although its cost was more than that of the original Hoechst 33258 system [7], its total expense, less than US$5 per measurement, was far more economical than that of real-time PCR. This could open certain feasibility to access many samples at one time. Our method demonstrated several merits in using the electrochemical approach especially on its rapidity and simplicity, on the characteristic of quantification (which required no sophisticated and expensive devices, less dependence on expensive reagents and instruments yet answering point of care test), and on its cost effectiveness. Its quantitative application could also expedite the need to answer a threshold analysis which had been applied for GMO content determination.

6. Conclusion

We have demonstrated the use of 5’ thiolation of forward primer and 5’ biotinylation of reverse primers for PCR amplification of trace target signals in food samples, the use of S1 nuclease treatment to reduce non-target signals after the amplification, and the Fc–Stv binding to biotin portions of DNA products for specific target DNA signal accumulation. After mediated allocation of signals on the surface of a gold electrode by thiol groups, the intensity of signals of redox reactions induced by the aggregation of DNA products at the electrode could be obtained using Hoechst 33258. This system allowed the direct detection of target DNAs at a maximum efficiency while requiring no laborious immobilization of probes or DNAs onto the surface of the electrode, providing better mean to quantify DNA while answering the need for point of care testing.

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