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Homogeneous Electrochemical Biosensor for Melamine Based on DNA Triplex Structure and Exonuclease III-assisted Recycling Amplification

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Abstract

Abasic site (AP site) in the triplex structure can recognize specific target with high selectivity. In this study, this character was first applied to develop a simple, sensitive, and selective homogeneous electrochemical biosensor for melamine determination. The assay combines the advantage of the high selectivity of the DNA triplex structure containing an AP site to melamine and high efficiency of exonuclease (Exo) III-assisted recycling amplification. DNA-1 (T1), DNA-2 (T2), poly[dA] probe containing an AP site (8A), and methylene blue-labeled DNA probe (dMB probe) were carefully designed. Melamine can specifically locate in the AP site through hydrogen bonding interaction between thymine and melamine to make T1, T2, and 8A close to each other, therefore forming a stable T-melamine-T DNA triplex structure. Under the optimal conditions, the differential pulse voltammetric (DPV) response had a linear relationship with the logarithm of melamine concentration in the range of 1 nM ~ 0.5 μM. The developed biosensor has been successfully applied to detect the migration of melamine from melamine bowl. Result showed that the migration in 4% acetic acid solvent was the largest, which is similar to that detected by high performance liquid chromatography. This homogeneous electrochemical sensor may have a potential prospect in detecting melamine in dairy products and migration of melamine from multi-category food packaging or application materials.

Introduction

Triplex structure is formed through sequence-specific Hoogsteen or hydrogen bond between single-stranded oligonucleotides and purine bases in the major groove of a target duplex and has attracted great attention because of its potential application in developing new molecular biology tools.[1-4] The DNA triplex structure is similar to the aptamer and has many excellent properties in biosensor design, such as high selectivity, low cost, simple synthesis, reusability, and high affinity and flexibility. Abasic site (AP site), one of the most common form of DNA damage, can be artificially incorporated into DNA or RNA duplexes to provide a hydrophobic vacancy for a ligand to bind to the nucleobase through pseudo-base pairing.[5,6] Specially designed AP site in the triplex structure can recognize specific target with high selectivity. For example, a molecular beacon containing an AP site has been designed, and melamine can locate in this AP site to form a DNA triplex structure through hydrogen bonding interaction between thymine and melamine. This triplex molecular beacon with AP site has good ability to recognize melamine selectively.[7] Furthermore, a highly selective fluorescence biosensor for melamine on the basis of this principle has been developed by the same group.[8]

Melamine, a nitrogen-rich toxic contaminant is also a synthetic chemical compound widely used in the manufacture of melamine formaldehyde resin in chemical industries. Because of possessing good heat-resistance and durability, it can be used in the manufacturing of plastic-ware such as melamine dishware.[9] Many

studies have shown that products of melamine formaldehyde resin such as utensils or food contact materials can decompose and release melamine monomer or derivatives into food under extreme heat, acidity, and polarity solution. Consumption of melamine contaminated products can cause kidney stones and other renal problems. So, many countries strictly stipulated the migration limit of melamine (0.4 mg/dm^2 in EU; 0.2 mg/dm^2 in China) from food contact materials. Therefore, it is necessary to develop some specific and sensitive biosensors for detecting the migration of melamine from food contact materials.

Several analytical methods based on chromatography,[10-17] optical method,[18-22] and immunoassay principle [23,24] have been attempted to detect melamine. Electrochemical detection has the advantages of high sensitivity, low cost, and easy operation.[25] Many electrochemical sensors have been developed for melamine detection. For example, a simple electrochemical sensor based on molecularly imprinted polymer of para-aminobenzoic acid (pABA) has been proposed for melamine detection.[26] An electrochemical sensor has been engineered for melamine by using ferricyanide as a redox indicator and a gold nanoparticles modified indium tin oxide electrode as base materials.[27] These electrochemical biosensors show the character of high sensitivity, but the electrode modification is a need in these electrochemical biosensors. These processes are complicated and time-consuming, which may affect the reproducibility of the biosensor. So it is still necessary to search some simple methods for melamine detection.

Indium tin oxide (ITO) electrode surface can be negatively charged after simple treatment,[28] which can repel DNA because the molecular skeleton of DNA contains the negatively charged phosphate. There is differential electrostatic repulsion between long-chain DNA and short-chain DNA toward a negatively charged ITO electrode surface. In view of these characters, many simple but sensitive homogenous electrochemical biosensors have been developed for diverse targets.[29-35] These biosensors not only retain the high sensitivity of the electrochemical detection but also avoid the tedious and time-consuming electrode modification steps, making the processes simpler and easier.

In this study, we first constructed an electrochemical biosensor for melamine, which combines the advantages of the high selectivity of the DNA triplex structure containing an AP site to melamine with the convenience and simplicity of modification-free homogeneous electrochemical detection. Furthermore, signal amplification strategy with the help of exonuclease III (Exo III) was coupled to enhance the performance of the biosensor. The proposed biosensor was eventually employed to detect the migration of melamine from melamine bowl.

Experimental Section

Chemicals and Reagents. All oligonucleotides were synthesized (>97%) by Shanghai Sangon Biotechnology Co., Ltd (Shanghai, China), and their sequences are shown below:

DNA-1 containing poly[dT] (T1): 5′-*ACG TA*A TCG A**TT TTT TTT TTT** GAG A-3′ (italic bases denote the region of hybridization with dMB probe; bold bases constitute the DNA triplex)

DNA-2 containing poly[dT] (T2):5′-TAG T**TT TTT TTT TTT** CG*C CCT TCC A*TA CGA GA-3′ (bold bases constitute the DNA triplex; italic bases denote the region of hybridization with dMB probe)

Poly[dA] probe containing an abasic site (8A): 5′-AAAAXAAA-3′ (X denotes the AP site (Spacer C3))

Single-strand DNA probe labeled with methylene blue (dMB probe):5′-CTG TGT AGC AGC *TGG AAG GGT ACG T*-MB-3′ (italic bases denote the region of hybridization with T1 and T2)

Label-free DNA with the same sequence of dMB probe (L-DNA): 5′-CTG TGT AGC AGC *TGG AAG GGT ACG T*- 3′ (italic bases denote the region of hybridization with T1 and T2)

10× NEBuffer 3 (50 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, pH 7.9) was purchased from New England Bio Labs (Beijing, China). Exo III was purchased from Thermo Fisher Scientific (Shanghai, China) and used directly without further purification. Melamine was purchased from Aladdin Reagents Co., Ltd. (Shanghai, China). Melamine bowl was purchased from local supermarket (Fuzhou, Fujian). All other chemicals were of analytical grade. Ultrapure water (Direct-Q3 UV system, Millipore, resistance 18.2 MΩ.cm) was used throughout the

project.

Electrode Pretreatment & Instrument. Before each electrochemical measurement, the ITO electrode was sequentially sonicated in an Alconox solution (20 g/L) for 15 min, propan-2-ol for 15 min, and ultrapure water for 15 min.[36] After these cleaning procedures, a negatively charged working electrode surface can be achieved. The working area of the ITO electrode was 5 mm × 3 mm. Differential pulse voltammetric (DPV) measurements were conducted on a computer controlled CHI660a electrochemical system (Shanghai, China). The electrochemical system contains an ITO working electrode and two platinum wires working as auxiliary and reference electrodes, respectively. The DPV signals were recorded ranging from -0.6 to 0 V.

Homogeneous Electrochemical Detection of Melamine. Before the electrochemical measurements, 50 μL of reaction solution containing 1× NEBuffer 3, 0.5 μM dMB probe, 0.1 μM T1, 0.1 μM T2, 0.1 μM 8A, 20 U of Exo III was mixed with different concentrations of melamine firstly and then incubated at 25 ℃ for a certain period of time. Then, the DPV signal of the mixture was recorded. Each sample was detected for five times, and the average value was calculated for quantitative analysis.

Gel Electrophoresis. Polyacrylamide gel electrophoresis (20%) was carried out to verify the formation of the hybrid product in the proposed biosensor. dMB probe in native polyacrylamide gel electrophoresis (PAGE) assay was replaced by label-free single-stranded DNA with the same sequence (L-DNA), enabling the fluorescence

intensity of hybrid products unaffected in the gel. The concentrations of all DNA sequences were 8 μM and the concentration of melamine was settled as 10 μM. All reaction samples were incubated at 25 ℃ for 2 h. Then the products of the reaction were mixed with Super Green (ultimate concentration of 100×) and incubated at room temperature for 15 min. Project was performed in 1× Tris-borate-EDTA (TBE) buffer at a constant voltage of 80 V for 2.5 h at room temperature. The gels were photographed by gel image system.

Detection of the Migration of Melamine from Melamine Bowl. Acetic acid (4%), ethanol (10%), and distilled water were selected as the simulating solutions. Melamine samples were extracted by using liquid-solid extraction. Simulating solution (250 mL) was pre-heated and then poured into melamine bowl for incubation (70 ℃, 4 h). Then three kinds of soaking solutions were separately recycled and concentrated. Then concentrated solution of each sample (5 μL) was used for the detection of migration of melamine from the bowls.

Results and Discussion

Principle of the Homogeneous Electrochemical Biosensor for Melamine. Scheme 1 illustrates the principle of the developed homogeneous electrochemical biosensor for melamine determination on the basis of Exo III-assisted recycling amplification. T1 and T2, both containing a short poly[dT] sequence, can hybridize with the 8A-containing poly[dA] sequence to form a DNA triplex structure.[7] The dMB probe

consisting of 25 bases was designed to be complementary with the light yellow part of T1 and T2 and had a methylene blue tag at its 3′ terminus. In the absence of melamine, simple addition of 8A, T1, and T2 cannot form a stable T-melamine-T triplex owing to the limited affinity. The dMB probe and T1 were designed to be only 5 bases complementary and could not form a stable double-stranded structure, so the dMB probe is free in the solution and cannot be digested by Exo III (especially degrades dsDNA from blunt ends in the direction from the 3′ to the 5′ end).[37,38] Given the strong electrostatic repulsion between the dMB probe and the negatively charged ITO electrode surface, the dMB probe cannot diffuse easily to the electrode surface. Thus, only a weak electrochemical signal can be detected.

Melamine can locate in the AP site of 8A and bind to the opposite thymine nucleobases through T-melamine-T hydrogen bonding. So T1, T2, and 8A can form a stable T-melamine-T DNA triplex structure through hydrogen bonding after the addition of melamine. Then the dMB probe can recognize and hybridize with T1 and T2 to form a T-shaped DNA structure, which includes a blunt end and serves as the substrate of the Exo III. So the dMB probe can be specifically hydrolyzed by Exo III, resulting in the formation of short MB-labeled mononucleotides fragments (MB-MFs) and the releasing of the T-melamine-T DNA complex. Unlike the dMB probe with large size, the released MB-MFs are smaller and contain less negative charges. Thus the electrostatic repulsion between the negatively charged ITO working electrode and MB-MFs is much smaller than that with the dMB probe. Therefore, the MB-MFs can

diffuse easily to the negatively charged ITO surface, and an increased electrochemical signal can be detected. At the same time, the released T-melamine-T DNA complex can trigger a new cycle of hybridization, cleavage, and release, thus plenty of MB-MFs can be generated; this further enhances the detected electrochemical signal and therefore achieves the signal amplification. By this mean, a sensitive homogeneous electrochemical biosensor for melamine can be developed.

Feasibility Test. DPV measurement was carried out to investigate the feasibility of the proposed biosensor. As shown in Fig. 1A, in the presence of dMB probe only, a weak DPV signal was detected (curve a). This result indicates that the electrostatic repulsion between the negatively charged ITO electrode and dMB probe is strong, thereby preventing the methylene blue from reaching the surface of the electrode. With the addition of Exo III and T1, Exo III and T2, or Exo III, T1, and T2, respectively, the electrochemical signals were seldom changed (curve b, c, and d, respectively), suggesting that the substrate of Exo III is not formed without 8A and melamine. With the further addition of 8A, slight DPV signal enhancement appeared (curve e). The reason lies in that sequence 8A itself still cannot hybridize with T1 and T2 to form a stable triplex structure without melamine, so the dMB probe cannot hybridize with the triplex structure and the cleavage of Exo III is forbidden. However, once melamine was added to the above mentioned system, an obvious enhanced electrochemical response was observed (curve f). The probable explanation is that a DNA triplex structure is steadily formed, which can hybridize with dMB to form a

T-shaped DNA structure. The dMB probe in the T-shaped DNA structure can be digested by Exo III, and DNA triplex structure present in the solution can hybridize with another dMB to cause the cycling of digestion by the Exo III, and large amounts of oligonucleotide fragments with less negative charges are generated, which can easily diffuse to the negatively charged ITO surface and cause the strong signal. However, a noticeable decrease of the DPV signal was observed without the addition of Exo III (curve g). These results indicate that the electrochemical signal amplification results from the Exo III-assisted cycling cleavage of the T-melamine-T complex.

PAGE was used to further verify the mechanism of the designed system. As shown in Fig. 1B, the incorporation of T1, T2, and 8A was so weak in the absence of melamine that nearly no T-melamine-T complex formed (lane b). The addition of L-DNA, which replaces the dMB probe, could not result in the formation of macromolecule (lane c). However, T-melamine-T complex with macromolecule weight was observed obviously after the addition of melamine (lane d). With the further addition of L-DNA, the hybridized product of L-DNA and T-melamine-T complex with high molecular weight appeared (lane e). But, assembly products were almost invisible when only T1, T2, and L-DNA existed in the system (lane f). These results clearly verify the feasibility of the developed biosensor.

Optimization of the Experimental Conditions. The concentration of the dMB probe causes great effect to the performance of the proposed biosensor. Low concentration

of dMB probe might result in insufficient digestion cycles, leading to the low sensitivity of the biosensor. Whereas excessive amounts of dMB probe might lead to a high background signal, limiting the sensing performance, so the concentration of dMB probe was optimized firstly. The concentrations of the melamine, T1, T2, and 8A were 0.1 μM, and the amount of Exo III was fixed at 20 U. Fig. 2A reveals the DPV signal firstly rose with the increase of dMB probe concentration and then gradually reached a plateau after 0.5 μM. The background signal also enhanced when the concentration of dMB probe was higher than 0.6 μM. The biggest difference of electrochemical signal appeared at 0.5 μM. Therefore, 0.5 μM dMB probe was chosen as the optimal condition in this work.

The efficiency of signal amplification is mainly derived from Exo III-catalyzed digestion for dMB probe in the T-shaped DNA triplex structure. It is well known that the catalytic ability of Exo III is closely related to the reaction temperature, digestion time, and dosage. Therefore, these conditions also need to be optimized. As presented in Fig. 2B, the electrochemical signal increased with the extension of digestion time and reached a plateau after 60 min. Therefore, 60 min was selected as the optimal digestion time for the following study. Fig. 2C shows the effect of the reaction temperature on the electrochemical signal. The DPV signal increased as the incubation temperature rose up to 25 °C and then decreased with the further increase of temperature. This is likely because if the temperature is too high and reaches the unwinding condition of T-melamine-T triplex DNA structure (25 °C), the triplex DNA

structure becomes unstable and fails to form. The dMB probe cannot hybridize with T1 and T2 to form a T-shape structure, so no MB-MFs are produced, causing the decrease of signal. When the temperature was up to 45 °C, the DPV signal decreased sharply. This is probably because the activity of Exo III is inhibited when the incubation temperature is over the optimal temperature of the enzyme (37 °C) and even becomes inactive (70 °C). So, 25 °C was selected as the reaction temperature in this study. Finally, the effect of the enzyme dosage was also studied. The electrochemical signal firstly increased with the increment of Exo III dosage and reached a plateau after 20 U (Fig. 2D). Therefore, 20 U was selected as the optimal reaction dosage for this work.

Calibration Curve for Melamine Detection. The DPV responses at different concentrations of melamine were recorded under the optimized conditions. As shown in Fig. 3A, the DPV signal increased with the increase of melamine concentration. The peak current had a good linear relationship with the logarithm of melamine concentration in the range of 1 nM ~ 0.5 μM (Fig. 3B). The regression equation can be expressed as follow:

$$\Delta I_p/\mathrm{nA} = -41.303 \times \mathrm{Log}C/\mathrm{M} - 409.174,\ R^2 = 0.990 \quad (1)$$

where ΔI_p is the difference of the peak currents detected with and without melamine, C is the concentration of melamine, and R^2 is the correlation coefficient. The detection limit was calculated to be 0.43 nM (S/N = 3). Compared with the previously reported fluorescent methods based on T-melamine-T DNA triplex structure [7] and

electrochemical methods based on the conversion of an electroactive complex [39] or gold nanoparticle-modified ITO electrode [27] for melamine detection, the developed biosensor has lower detection limit and higher sensitivity. This high sensitivity can be attributed to the high intrinsic reaction efficiency of immobilization-free approach, as well as the excellent enzyme-assisted signal amplification mechanism.

Selectivity Study. To evaluate the selectivity of the developed electrochemical melamine sensor, several highly similar structural analogs of melamine (including adenine, thymine, guanine, cytosine, and cyanuric acid) and some potential coexistent species and amino acids (including glucose, lactose, potassium ions, sodium ions, magnesium ions, calcium ions, cysteine, histidine, glycine, lysine, tryptophan, and isoleucine) in real samples were chosen as interferences at 100-fold concentration of melamine. Weak electrochemical signals were observed in the presence of interferences only, whereas the addition of melamine would lead to a strong signal (Fig. 4). These results indicate the good selectivity of this developed method.

Migration of Melamine from Melamine Bowl. The migration of melamine from melamine bowl was measured by the developed electrochemical sensor. Table 1 lists the comparative determination results for these samples by high performance liquid chromatography (HPLC) and the proposed electrochemical method. The results from the proposed biosensor are consistent with that from HPLC. The highest migration of melamine from the melamine bowl appeared in 4% acetic acid simulation solvent incubated at 70 °C for 4 h. The relative errors of two methods were less than ±10%.

The detected migrations of melamine monomer by the developed electrochemical sensor in 10% ethanol simulation solvent, water simulation solvent, and 4% acetic acid simulation solvent were 0.081 ± 0.004, 0.109 ± 0.005, and 0.198 ± 0.008 mg/dm^2, respectively. These outcomes further suggest that the developed biosensor can be applied to test the migration of melamine in real samples with satisfactory results.

Conclusions

In summary, a simple, sensitive, and homogeneous electrochemical method has been developed for melamine determination, combining the advantage of Exo III-assisted recycling amplification, the high recognize ability of AP site-containing DNA triplex structure, and the character of differential electrostatic repulsion between long-chain DNA and short-chain DNA toward a negatively charged ITO electrode surface. This biosensor system exhibits good selectivity and high sensitivity. To the best of our knowledge, this is the first example which uses AP site-containing DNA triplex to achieve electrochemical detection for melamine in homogeneous phase, with the detection limit of 0.43 nM. Compared with other methods for the melamine analysis, homogeneous electrochemical strategies avoid the tedious and time-consuming steps, facilitating the experimental processes. The proposed strategy has also been successfully demonstrated its practical application for the detection of melamine migration from melamine bowl.

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Figures and Captions

Scheme 1 Schematic illustration of the proposed homogeneous electrochemical biosensor for melamine detection based on DNA triplex structure and Exo III-assisted recycling amplification.

Figure 1 (A) DPV responses under different conditions: (a) dMB probe; (b) dMB + T1 + Exo III (c) dMB + T2 + Exo III; (d) dMB + T1 + T2 + Exo III; (e) dMB + T1 + T2 + 8A + Exo III; (f) dMB + T1 + T2 + 8A + melamine + Exo III; (g) dMB + T1 + T2 + 8A + melamine. The concentrations of dMB, T1, T2, 8A and melamine were 0.5, 0.1, 0.1, 0.1, and 1 μM, respectively, and the amount of Exo III was 20 U. **(B)** PAGE analysis under different conditions: Lane a: marker; Lane b: T1 + T2 + 8A; Lane c: T1 + T2 + 8A + L-DNA; Lane d: T1 +T2 + 8A + melamine; Lane e: T1 + T2 + 8A + melamine + L-DNA; Lane f: T1 + T2 + L-DNA.

Figure 2 The effect of the **(A)** dMB probe concentration; **(B)** digestion time; **(C)** incubation temperature of Exo III; **(D)** dosage of Exo III. [dMB probe] = 0.5 μM, [T1] = 0.1 μM, [T2] = 0.1 μM, [8A] = 0.1 μM, [Exo III] = 20 U, [Melamine] = 0.1 μM.

Figure 3 (A) DPV responses of reaction mixtures containing 0.5 μM dMB probe, 0.1 μM T1, 0.1 μM T2, 0.1 μM 8A, and varying concentrations of melamine. **(B)** The current increment plotted against concentration of melamine. From a to h: no target, 1 nM, 5 nM, 10 nM, 50 nM, 0.1 μM, 0.5 μM, 1μM.

Figure 4 The specificity of the proposed assay. From a to r: melamine, glucose, lactose, adenine, thymine, guanine, cytosine, Ca^{2+}, K^{+}, Na^{+}, Mg^{2+}, cyanuric acid, cysteine, histidine, glycine, lysine, tryptophan, and isoleucine. The concentrations of melamine and other substances are 0.1 and 10 μM, respectively.

Table1 Detection of the migration of melamine from melamine bowl (n = 5).

Scheme 1

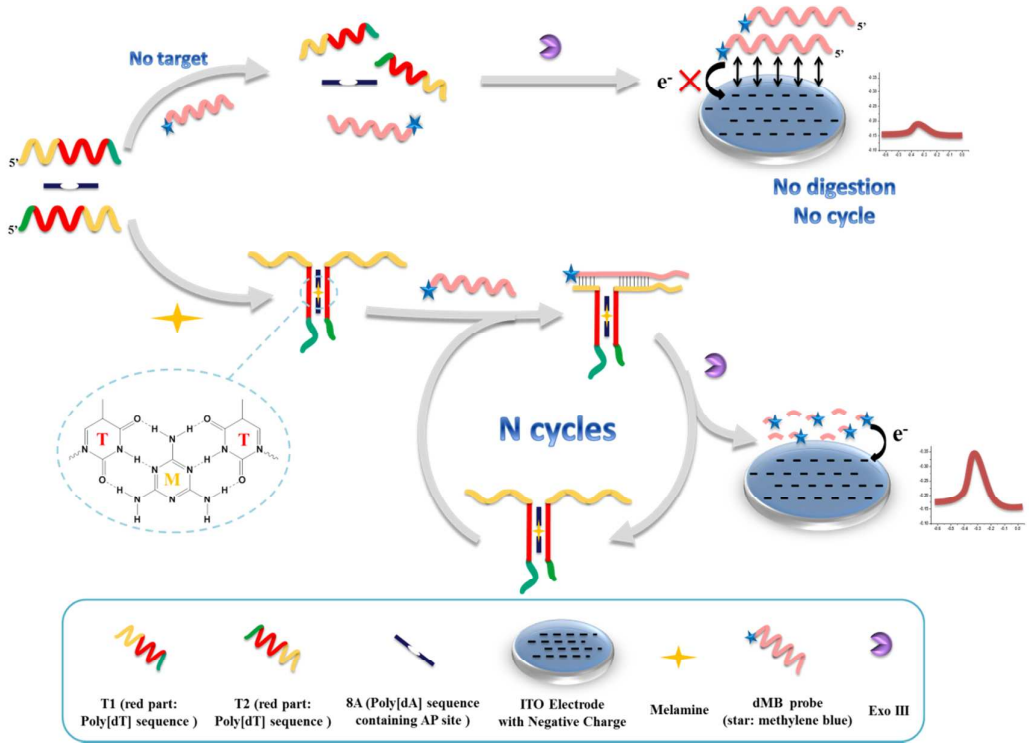
No target
5'
5'
e-
5'
5'
No digestion
No cycle
T
M
T
N cycles
e-
T1 (red part: Poly[dT] sequence)
T2 (red part: Poly[dT] sequence)
8A (Poly[dA] sequence containing AP site)
ITO Electrode with Negative Charge
Melamine
dMB probe (star: methylene blue)
Exo III

Figure 1

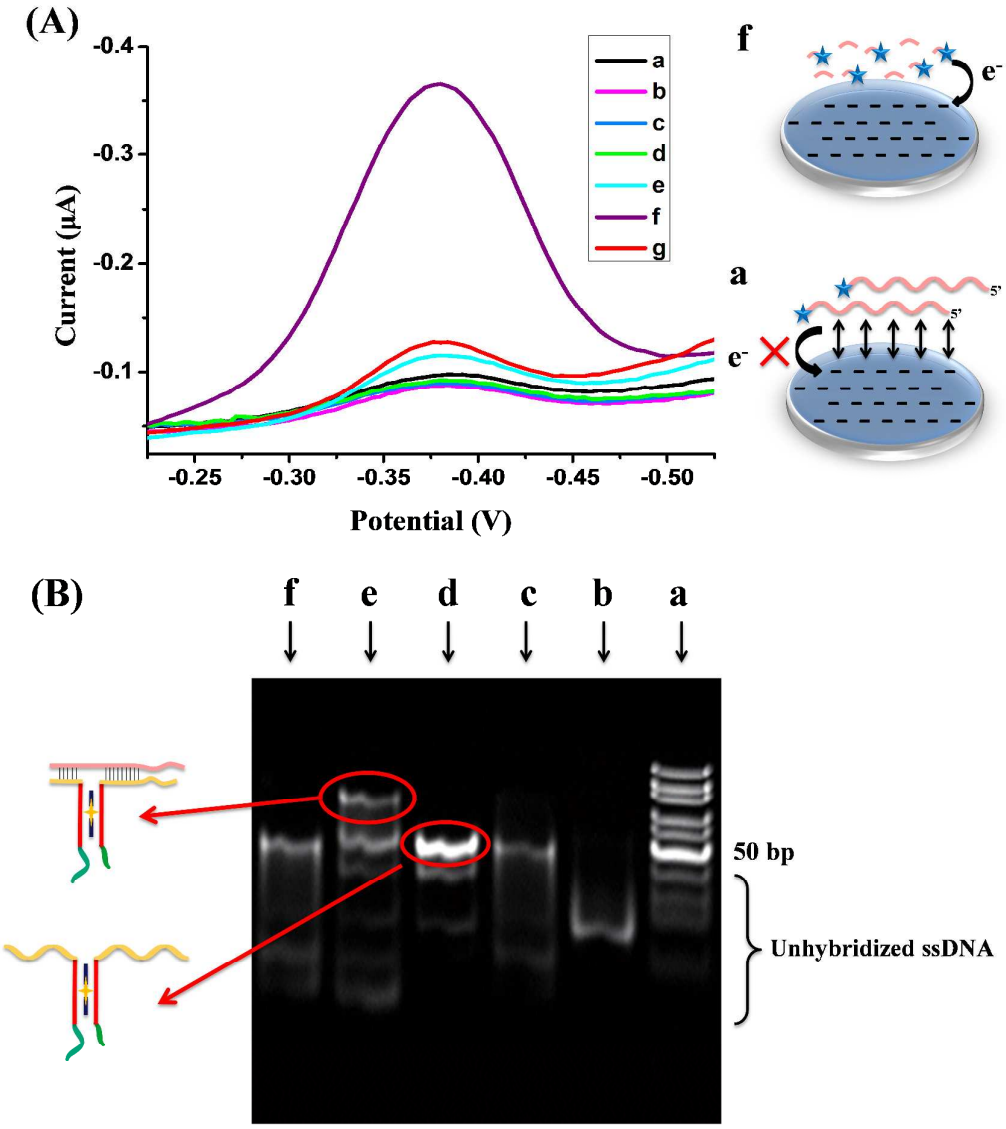
(A)
Current (μA)
-0.4
-0.3
-0.2
-0.1
-0.25
-0.30
-0.35
-0.40
-0.45
-0.50
Potential (V)
a
b
c
d
e
f
g
f
e⁻
a
e⁻
5'
5'
(B)
f
e
d
c
b
a
50 bp
Unhybridized ssDNA

Figure 2

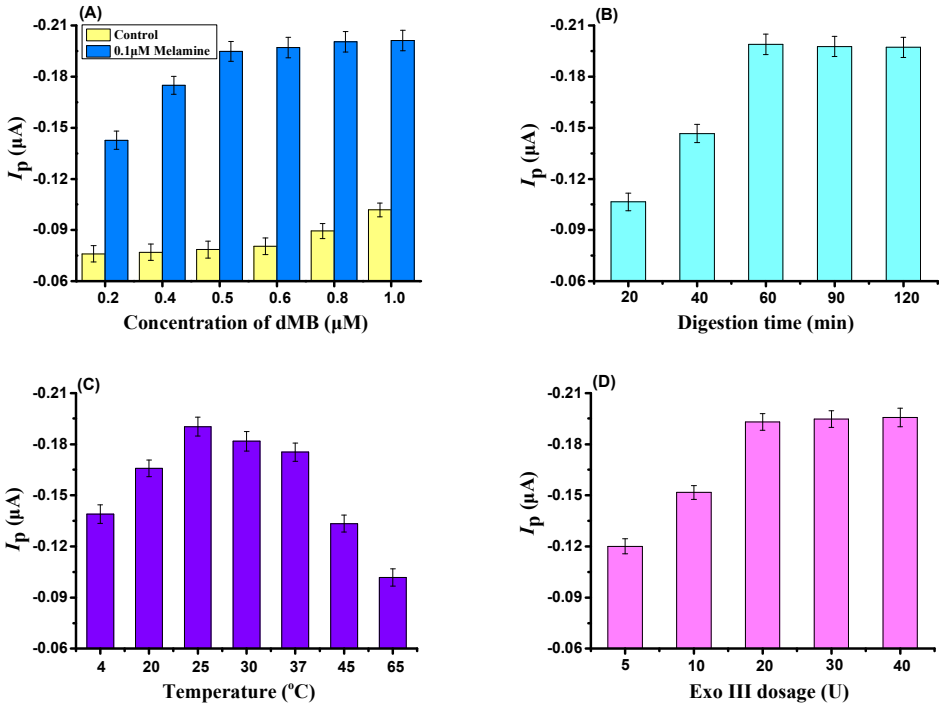
(A)
Control
0.1μM Melamine
I_p (μA)
-0.21
-0.18
-0.15
-0.12
-0.09
-0.06
0.2
0.4
0.5
0.6
0.8
1.0
Concentration of dMB (μM)
(B)
I_p (μA)
20
40
60
90
120
Digestion time (min)
(C)
I_p (μA)
4
20
25
30
37
45
65
Temperature (°C)
(D)
I_p (μA)
5
10
20
30
40
Exo III dosage (U)

Figure 3

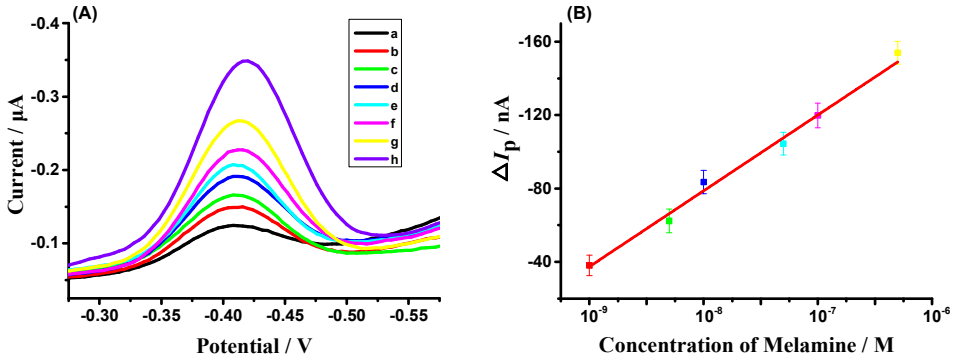
(A)
Current / μA
-0.4
-0.3
-0.2
-0.1
-0.30
-0.35
-0.40
-0.45
-0.50
-0.55
Potential / V
a
b
c
d
e
f
g
h
(B)
ΔIp / nA
-160
-120
-80
-40
10-9
10-8
10-7
10-6
Concentration of Melamine / M

Figure 4

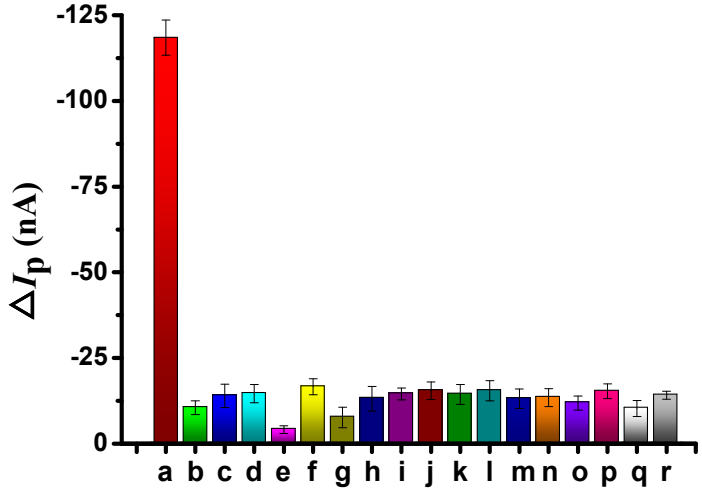

Table 1 Detection of the migration of melamine from melamine bowl (n = 5)

Sample	Detected (μM)	Detected by HPLC (μM)	Relative error (%)	RSD (%)	Melamine migration (mg/dm^2)
10% ethanol	3.738	3.467	7.8	4.6	0.081 ± 0.004
water	5.040	5.313	-5.1	4.7	0.109 ± 0.005
4% acetic acid	9.212	8.674	6.2	4.3	0.198 ± 0.008

For TOC only: