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A DNA sequence-specific electrochemical biosensor based on alginic acid-coated cobalt magnetic beads for the detection of *E. coli*

Ping Geng[a], Xinai Zhang[a], Yingqiao Teng[a], Ying Fu[a], Lili Xu[a], Min Xu[b,*], Litong Jin[a], Wen Zhang[a,*]

[a] Department of Chemistry, East China Normal University, 3663th Zhongshan Rd. (N), Shanghai 200062, PR China
[b] Shanghai Key Laboratory of Magnetic Resonance, Department of Physics, East China Normal University, Shanghai 200062, PR China

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ABSTRACT

A new type of DNA sequence-specific electrochemical biosensor based on magnetic beads for the detection of *Escherichia coli* is reported in the present work. Alginic acid-coated cobalt magnetic beads, capped with 5′-(NH_2) oligonucleotide and employed not only for magnetic separation but also as the solid adsorbent, were used as DNA probes to hybridize with the target *E. coli* DNA sequence. This assay was specific for *E. coli* detection depending on the *uid A* gene, which encodes for the enzyme β-D-glucuronidase produced by *E. coli* strains. When daunomycin (DNR) was used as DNA hybridization indicator, the target sequences of *E. coli* hybridized with the probes resulted in the decrease of DNR reduction peak current, which was proportional to the *E. coli* concentration. The optimization of the hybridization detection was carried out and the specificity of the probes was also demonstrated. This DNA biosensor can be employed to detect a complementary target sequence for 3.0×10^{-10} mol/L and denatured PCR products for 0.5 ng/μL. The linear range of the developed biosensor for the detection of *E. coli* cells was from 1.0×10^2 to 2.0×10^3 cells/mL with a detection limit of 50 cells/mL. After a brief enrichment process, a concentration of 10 cells/mL *E. coli* in real water samples was detected by the electrochemical biosensor.
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1. Introduction

Escherichia coli (*E. coli*) is a typical inhabitant in the intestinal tracts of humans and warm-blooded animals, which is often preferred as an indicator organism because it is specific for water pollution and reliably reflects fecal contamination (Mittelmann et al., 2002; Min et al., 2002; Rompre et al., 2002). Some strains of *E. coli* can cause diarrhea, urinary tract infections, inflammations and peritonitis in immunosupressed patients as children and elderly people (Gfeller et al., 2005). As a consequence, the presence of *E. coli* in drinking water is considered as a possible threat or indicator of microbiological water quality deterioration. Therefore, a sensitive and specific determination of *E. coli* is required for safety and hygiene reasons.

Conventional microbiological methods (Clesceri et al., 1998) for *E. coli* detection are mainly based on cultures grown on differential agar media followed by counting the number of target organisms in the samples. The time-consuming and laborious process drawback of the conventional methods increases the motivation for the development of sensitive and specific techniques aiming at the detection of *E. coli*. In the past years, several methods have been proposed such as optical, biochemical, electrochemical assays and so on (Su and Li, 2004; Ruan et al., 2002; Serra et al., 2005; Kulagina et al., 2005; Chang et al., 2001). Among these techniques, DNA biosensor has emerged as a promising alternative for microbial detection due to the specificity of hybridization between the probe and the complementary target sequence (Shiraishi et al., 2007). However, the non-specific adsorption in the hybridization system increases the strong demand for development of a sensitive and specific DNA biosensor.

Nowadays, magnetic separation has become an interesting tool for bioassays because the magnetic beads enable the isolation or extraction of a target molecule or substance by external magnetic field (Palecek and Fojta, 2007; Ravindranath et al., 2009; Chen et al., 2008). Due to the good biocompatibility and adequate functional groups for chemical fixation, magnetic beads modified with various recognition elements can be used for specific bioaffinity capture of different molecules. They are employed not only for magnetic separation of support from the reaction mixture but also as the solid adsorbent (Taylor et al., 2000). Therefore, magnetic beads have been applied to many areas such as the immobilization of proteins and enzymes (Yang et al., 2004), bioseparation (Ito et al., 2005), immunoassay (Tanaka and Matsunaga, 2000), drug delivery (Neuberger et al., 2005) and biosensors (Liu et al., 2005).

In this paper, a sensitive and specific DNA electrochemical biosensor based on magnetic beads for *E. coli* detection was developed. The DNA sequence of the *uid A* gene, which was coding for

* Corresponding authors. Tel.: +86 21 62233509; fax: +86 21 62232627.
E-mail address: wzhang@chem.ecnu.edu.cn (W. Zhang).

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the enzyme β-D-glucuronidase produced by *E. coli* strains, was utilized for the specific detection of *E. coli* (Tryland and Fiksdal, 1998; Min et al., 2002; Rompre et al., 2002). Alginic acid is a natural carboxylic polysaccharide consisting of β-(1-4)-D-manuronic acid and α-(1-4)-L-guluronic acid (Prodelalova et al., 2004). The newly designed magnetic cobalt particles modified with alginic acid were used for isolation of microbial DNA of *E. coli*. The NH_2 labeled oligonucleotide DNA probe was immobilized to magnetic beads for magnetic separation of the conjugated samples. When daunomycin (DNR) was used as DNA hybridization indicator, the biosensor based on the designed specific DNA probes was invited to directly detect *E. coli* target sequences in complementary oligonuleotides, *E. coli* denatured PCR products and *E. coli* cell lysate. Target sequence hybridized with the probe resulted in a decrease of the reduction peak current of DNR. Since the decreased current was proportional to the concentration of the hybridized target sequences, the quantitative detection of *E. coli* was realized. This method enabled to detect a complementary target sequence for 3.0×10^{-10} mol/L and denatured PCR products for 0.5 ng/μL. The detection range of *E. coli* was from 1.0×10^2 to 2.0×10^3 cells/mL and the detection limit was 50 cells/mL. With preconcentration and pre-enrichment steps, this method possessed sufficient sensitivity to detect 10 cells/mL *E. coli* in real water samples.

2. Experimental

2.1. Reagents and instruments

1-Ethyl-3-(dimethylaminopropyl)-carbodiimide hydrochloride (EDAC) and Daunomycin (DNR) was purchased from Sigma Chemicals (St. Louis, MO, USA). Sodium alginate $(C_6H_7O_6Na)_n$ was obtained from Qingxi Chemical Technological Company (Shanghai, PR China). Imidazole, Cobalt (II) acetate tetrahydrate ($C_4H_6O_4Co{\cdot}4H_2O$), Sodium borohydride ($NaBH_4$), Hydrazine hydrate ($N_2H_4{\cdot}H_2O$) and Sodium hydroxide (NaOH), Ethanol (C_2H_5OH) are all purchased from Guoyao chemicals Company (Shanghai, PR China). All oligonucleotide fragments were purchased from Shenenergy Biocolor Biological Science and Technology Co. (Shanghai, PR China).

Electrochemical measurement was achieved with a CHI 832 Electrochemical Analyzer (CHI Instruments Inc., USA). The detection was carried out in a common electrochemical cell containing a glassy carbon working electrode, an Ag/AgCl (saturated KCl) reference electrode and a platinum counter electrode. High-resolution transmission electron microscopy (TEM) images were collected on a JEOL JEM-2010 TEM (JEOL Ltd., Japan). IR-spectra (Mexus 670 FT-IR spectrom-eter Nicolet, USA) were used to study the chemical bonds between magnetic nanoparticles and RCOOH or RCOOH and DNA.

2.2. PCR amplification of target DNA

The sequence of polymerase chain reaction (PCR) primers for amplification of uid A region DNA from *E. coli* was designed according to Bej et al. (1991). The forward primer was 5′-AAAACGGCAAGA AAAAGCAG-3′ and the reverse primer was 5′-ACGCGTGGTTACA GTCTTGCG-3′. The specific oligonucleotides sequence of 5′-GGTAGCGTCGCATTACGAGATGTGGTGCGGCTTATGGACC-3′ was used as probe sequence with amino-group at 5′ end. The complementary sequence of 5′-GGTCCATAAGCCGCACCACATCTCG TAATGCGACGCTACC-3′ was used as the target sequence.

The standard PCR mixture (50 μL) contained 4 mM $MgCl_2$, 200 μM deoxynucleotide triphosphate, 14 nM primer, 50 ng of DNA template, and 2 U of Platinum Taq DNA polymerase. PCR amplification was performed using the following conditions: initial enzyme activation at 94 °C for 5 min, 28 cycles at 94 °C for 45 s, 55 °C for 45 s, 72 °C for 1 min, and final elongation at 72 °C for 5 min. Amplified DNA was visualized using gel electrophoresis.

2.3. Preparation of Co^0 particle embedded in sodium alginate

1 wt% sodium alginate aqueous solution was added into 5.0 mL 1.0 M aqueous solution of $CoC_4H_6O_4{\cdot}4H_2O$ by droplets with a needle tip under mild stirring. Gelation immediately occurred at the droplet surface, spherical capsules was formed. After stirring for 2 h, the gel beads were thoroughly washed with distilled water and filtration. Co^{2+}/alginate microcapsules were obtained.

Co^{2+}/alginate microcapsules were then transferred into a flask and dispersed in 12.0 mL ethanol. After adding 2.0 mL 0.1 M sodium borohydride solution, a mushy mixture of 8.0 mL hydrazine hydrate solution and 4.0 g sodium hydroxide was added dropwise into the suspension. The reaction system was then kept stirring at room temperature for 12 h.

2.4. Preparation of DNA probe/magnetic beads conjugate

Firstly, 2.0 mg imidazole was added into 100 μL magnetic beads suspended solution. After stirred for 10 min, 2.5 mg EDAC was added. The 1.0 o.d. of 5′-COOH capped oligonucleotide dissolved in 300 μL PBS solution with the addition of magnetic beads. The mixture was stirred for 10 h (at room temperature). Unbound oligonucleotides were removed easily by using a magnet under the cell for 3 min and washed with PBS solution three times, and then the magnetic beads/DNA probes were suspended in 300 μL PBS solution (pH 7.4).

5 μL of DNR solution ($c_{DNR} = 3.15 \times 10^{-3}$ mol/L) was added into 300 μL DNA probe solution under slowly stirring at 38 °C for 15 min. The non-bound DNR can be eliminated by washed with phosphate solution. The reduction peak current of DNR connected on DNA probe was measured by Differential Pulse Voltammetric analysis (DPV) scans from −0.30 V to −0.70 V (pulse width: 0.05 s, pulse period: 0.2 s, quiet time: 2 s).

2.5. Hybridization and detection

Prior to hybridization, the PCR samples and *E. coli* cells were first diluted in the hybridization buffer and then thermally denatured. The thermal denaturation was obtained by dipping the vial containing the PCR fragments in a boiling water bath at 95 °C for 10 min, followed by fast cooling in ice-water bath for 5 min (Sun et al., 2006; Courtney et al., 2006; Steichen et al., 2007). Similarly, heat-denatured samples were prepared for *E. coli* cells suspended in phosphate buffer (pH 7.0).

Hybridization experiments were carried out using synthetic oligonucleotides and amplified PCR targets, as well as *E. coli* cells. DNA probe was resuspended in 300 μL hybridization solution containing 0.2 mol/L PBS and 0.9 mol/L NaCl. Target DNA sequences were added with gentle stirring at 38 °C for 30 min and then washed with PBS to eliminate non-specific absorbed ssDNA. DNR reduction peak current was detected after hybridization by DPV scans from −0.30 V to −0.70 V (pulse width: 0.05 s, pulse period: 0.2 s, quiet time: 2 s).

3. Results and discussion

3.1. Characterization of magnetic beads and DNA probe

The size of alginic acid-coated magnetic cobalt particle was determined by Transmission Electron Microscope. It can be seen that the particles are quasi-spherical, and the diameters are about

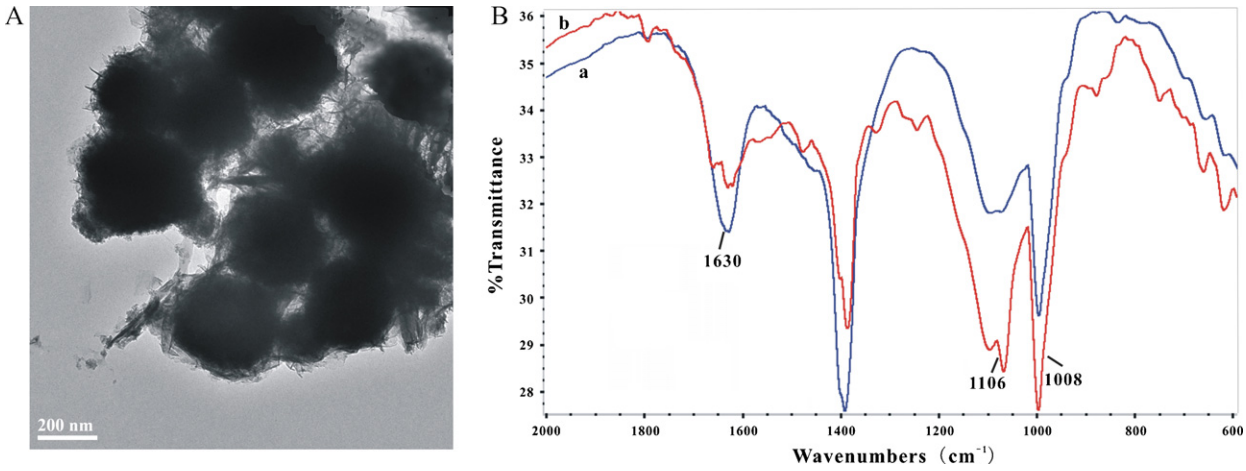

Fig. 1. (A) TEM image of magnetic cobalt particles modified with alginic acid. (B) IR spectrum of DNA probe: (a) bare COOH-coated magnetic particles, and (b) magnetic beads after conjugated with oligonucleotides (range: 2000–600 cm^{-1}, temperature: 25 °C).

200 nm (Fig. 1A). The size distribution and zeta potential were also studied. We have successfully obtained magnetic particles with a narrow size distribution (Pdi: 0.134) and the measurement of zeta potential (−21 mV) indicated that the magnetic particles were stable in the PBS solution at pH 7.4 and in presence of 0.9 mol/L NaCl. Furthermore, the chemical bonds between cobalt particles and RCOOH or magnetic beads and DNA were confirmed by IR (Fig. 1B). In the spectrum of COOH-coated magnetic particles, an absorption peak at 1630 cm^{-1} represented the stretch vibration of C=O ($\upsilon_{C=O}$) in carboxylic group. So it is confirmed that the surface of the magnetic particles was coated by macromolecules. After conjugating with DNA, two strong absorption peaks were found at 1008 and 1106 cm^{-1}, representing the stretch vibration of $-CH_2-O-P$ (υ_{P-O-}). The IR spectrums demonstrate that DNA was conjugated on the magnetic beads. The electrochemical behavior of DNA probes or hybridized with target sequence was also made to further demonstrate that DNA was conjugated on the magnetic beads (Fig. 4).

3.2. Mechanism of detecting E. coli

In the highly specific hybridization detection system, the non-specific DNA adsorption is particularly difficult to avoid especially when one works with real samples. Carboxyl group-containing magnetic particles can be used for isolation of DNA sequences. The new strategy developed for *E. coli* detection based on DNA hybridization is illustrated in Fig. 2. The alginic acid-coated cobalt magnetic beads were synthesized for separation in the hybridization detection. The NH_2 labeled oligonucleotide DNA probe can be immobilized to the carboxyl on the alginic acid packed magnetic beads for magnetic separation of the conjugated samples. The *uid A* gene, which codes for the enzyme β-D-glucuronidase produced by *E. coli* strains, can be utilized for the specific detection of *E. coli*. This assay functions were based on hybridization between the DNA probes and the target complementary sequences of *uid A* gene in *E. coli* cells. The hybridization detection was accomplished via the reduction of adsorbed DNR, which was widely used as the DNA

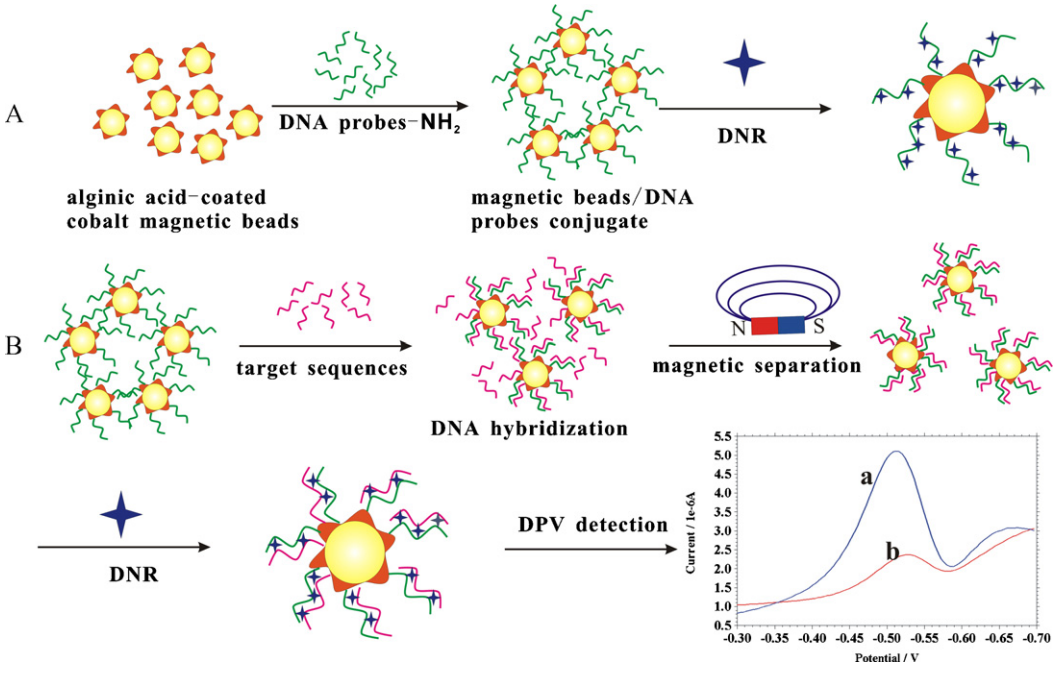

Fig. 2. Schematic representation of DNA hybridization detection based on the electrochemical biosensor.

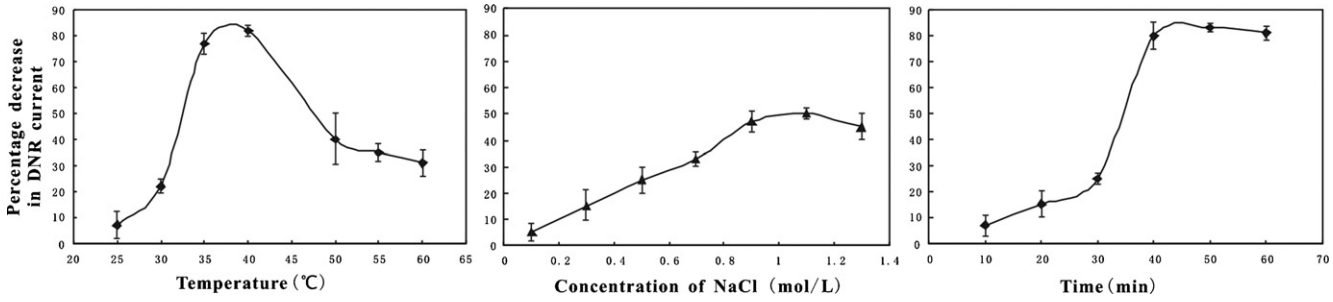

Fig. 3. Optimization of *E. coli* DNA hybridization detection: (a) the influence of temperature, (b) the influence of ionic strength, (c) the influence of time.

hybridization indicator. The current signal of DNR was first detected before hybridization (curve a). Then target sequence hybridized with the probe resulted in the decrease of the reduction peak current of DNR connected with probe (curve b). Since the decreased current was proportional to the concentration of the hybridized target sequences, the quantitative detection of *E. coli* was realized.

3.3. Optimization of E. coli DNA hybridization

Temperature, ionic strength and hybridization time are three factors influencing on the *E. coli* DNA hybridization (Cheng et al., 2005; Arora et al., 2007). As seen in Fig. 3a, when the temperature was lower than 30 °C, the current decrease of DNR was not obvious. While temperature rose from 30 to 50 °C, the hybridization efficiency gradually increased. But when the temperature was higher than 50 °C, the denaturation of double-stranded DNA may occur. Therefore, 40 °C was chosen for the detection of target sequence.

The tests for selecting optimal ionic strength in hybridization were made in 0.2 mol/L PBS solution (pH 7.4) with different concentration of NaCl in the range of 0.1–1.3 mol/L. A gradual increase in the current response was found with the increase of NaCl concentration in PBS solution. When NaCl concentration was above 0.9 mol/L, a plateau was observed (Fig. 3b). To achieve the highest response of DNA hybridization, NaCl of 0.9 mol/L was adopted for use in subsequent experiments.

Fig. 3c shows the DPV of the biosensor on hybridization with target complementary sequence with different hybridization time (10–60 min). When the hybridization time increased, the current response increased. When the hybridization time was over 40 min, the current response presented a slow increment. Hence, 40 min was selected as the optimal hybridization time.

3.4. Analysis of specificity for the detection of E. coli

The *uid A* gene, which encodes for the enzyme β-D-glucuronidase mainly produced by *E. coli* strains, can be utilized for the specific detection of *E. coli*. The selectivity of DNA probe based on magnetic beads was tested by the hybridization of DNA probe with different target DNA, such as complementary sequence, three-base mismatched sequence and non-complementary sequence. The intercalation of DNR is much more evident when hybridization occurs. Fig. 4 shows the current response of the DNA probes exposure to different target sequences. It clearly shows no hybridization between the DNA probes and the non-complementary sequences, because the peak current was almost the same with DNA probes (curve a and b). Compared with the three-base mismatched sequence (curve c), the complementary sequence depressed DNR reduction peak current heavily (curve d). The significant difference in these sequences confirmed that this protocol was of high selectivity.

The specificity of the biosensor for *E. coli* was determined using different bacteria, which were known to be potentially present in untreated water (i.e. *bacillus subtilis* and *microzyme*). Supplementary Fig. S1 indicates the change of current response after hybridization with different bacteria. When the DNA probes were hybridized with lysed *bacillus subtilis* and *microzyme* cells, the decrease of DNR current was less than 10%. However, when the hybridization was occurred between the probes and lysed *E. coli*

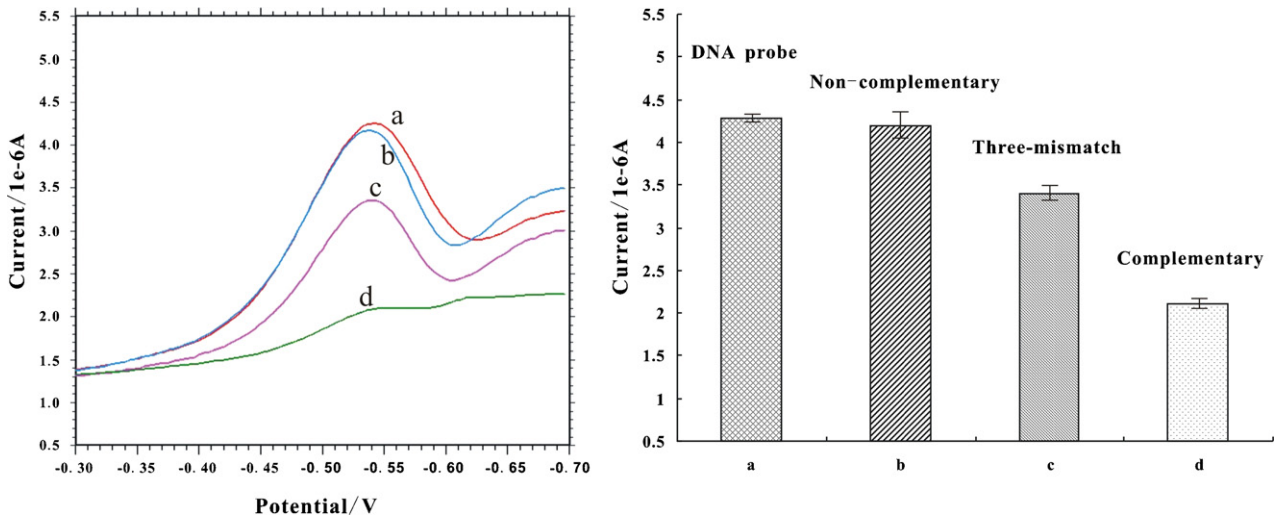

Fig. 4. DPV response of DNA probes after hybridization with target sequence (3×10^{-8} mol/L): (a) DNA probe blank (containing no-target) (b) with non-complementary sequences (c) with three-base mismatched in the sequence (d) with the complementary sequence.

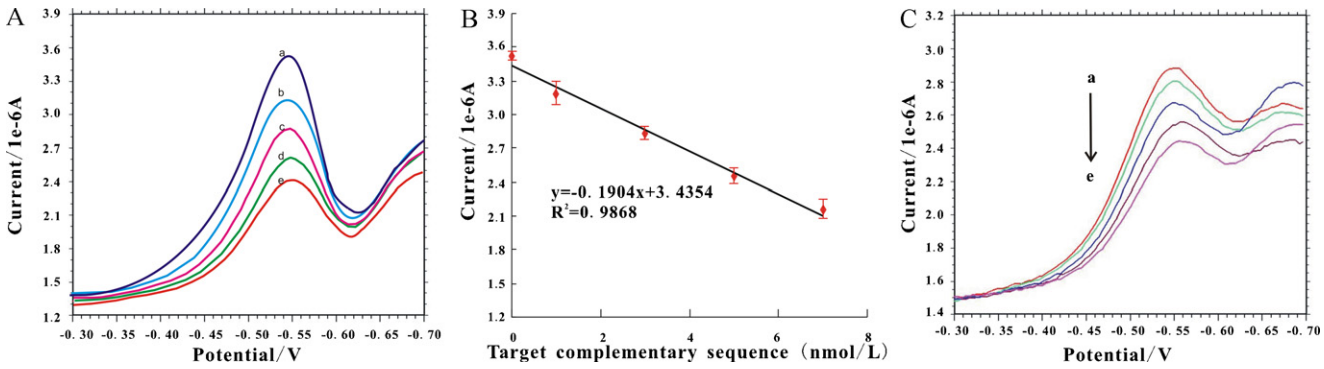

Fig. 5. (A) DPV curves of DNR in the probe hybridized with different concentration of complementary sequence (a) 0, (b) 1.0×10^{-9} mol/L, (c) 3.0×10^{-9} mol/L, (d) 5.0×10^{-9} mol/L, (e) 7.0×10^{-9} mol/L. (B) Calibration plot of DPV response vs. concentration of target sequences. (C) DPVs of DNA probe hybridized with heat-denatured *E. coli* cells: (a) 1.0×10^2, (b) 5.0×10^2, (c) 1.0×10^3, (d) 1.5×10^3 and (e) 2.0×10^3 cells/mL.

cells, a marked decrease (50–70%) was found. The results demonstrated that the decrease of DNR current reflected the hybridization between the probes and the target sequences. Therefore the specificity of the biosensor for *E. coli* is clearly shown. The presence of other related microorganisms did not provide significant interference.

3.5. The detection of E. coli target complementary sequences, E. coli PCR fragments and E. coli cells

The analytical performances of *E. coli* DNA detection were tested after 40 min of hybridization between the electrochemical biosensor and different concentrations of *E. coli* DNA sequences at 40 °C. The hybridization was first detected using the DNA probes and different concentrations of the complementary sequences. Fig. 5A indicates the DPV hybridization responses for increasing concentrations of target complementary sequence. Curves a–e represented the complementary sequences concentration of 0, 1.0×10^{-9}, 3.0×10^{-9}, 5.0×10^{-9} and 7.0×10^{-9} mol/L, respectively. A clear decrease of the DNR reduction signal was observed. This phenomenon was due to the DNA hybridization between the biosensor and the complementary DNA in solution. The current decrease of DNR was linear with the concentration of target complementary sequences with a correlation coefficient of 0.9868 (Fig. 5B). This observation suggested that the capture probes can be successfully used in *E. coli* DNA detection.

The DNA biosensor and the hybridization detection approach designed in our assay were utilized for *E. coli* PCR-amplified DNA and *E. coli* cells. With increasing the amount of PCR fragments, the reduction current of DNR decreases, indicating the hybridization occurs. The detection limit of this DNA biosensor for the denatured PCR products was 0.5 ng/μL. The DNA probe was also applied to detect lysed *E. coli* cells. Fig. 5C shows DPVs of DNA probes hybridized with lysed *E. coli* cells. With the increase in the concentration of *E. coli* cells, the DNR peak current decreased. A linear relationship between the decreased current and the *E. coli* concentration was found in a range of *E. coli* concentration from 1.0×10^2 to 2.0×10^3 cells/mL. When the concentration of lysed *E. coli* cells was lower than 50 cells/mL, there was almost no difference of the DNR current signal. Therefore, the electrochemical biosensor can be used to detect *E. coli* cells (50 cells/mL) without external reagent and the complete detection could be achieved in 2 h. In the experiment, the *E. coli* detection limit of 50 cells/mL was repeatedly determined using the proposed method 5 times and relative standard deviations (RSD) of 4.7% were acquired.

As *E. coli* remains an important indicator organism for the microbiological quality of water samples, the DNA electrochemical biosensor was applied to detect the real water samples. To get the achievement of the analysis, we modified the method by introducing a preconcentration step prior to the sample enrichment. The water sample containing *E. coli* yielded a response perfectly distinguishable from that of the blank sample. It was possible to detect *E. coli* concentration as low as 10 cells/mL in real water samples, which was consistent to the result obtained by plate count method (13 cells/mL).

4. Conclusion

In this paper, a novel sensitive method for the detection of *E. coli* was developed by DNA electrochemical biosensor coupled with magnetic separation technique. The method was based on the *uid A* gene, which encodes for the enzyme β-D-glucuronidase mainly produced by *E. coli* strains. The magnetic cobalt particles modified with alginic acid were used for isolation of microbial DNA of *E. coli*, which contributed to improve sensitivity and specificity. This method enabled to detect a complementary target sequence for 3.0×10^{-10} mol/L, denatured PCR products for 0.5 ng/μL and *E. coli* cells for 50 cells/mL. With preconcentration and pre-enrichment steps, this method possessed sufficient sensitivity to detect 10 cells/mL *E. coli* in real water samples. Therefore, the proposed technique could be successfully applied in the detection of *E. coli* for environmental monitoring and biomedical requirement.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.01.007.

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