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Diagnosis of *Helicobacter pylori* bacterial infections using a voltammetric biosensor

Suw Young Ly [a,*], Hai-Soo Yoo [b], Sung Hoon Choa [c]

[a] Biosensor Research Institute, Seoul National University of Science and Technology, Seoul, South Korea
[b] Korea Ocean R&D Institute, P.O. Box 29, Ansan 425-600, South Korea
[c] NIT Engineering Department, Seoul National University of Science and Technology, Seoul, South Korea

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ABSTRACT

The voltammetric assay of *Helicobacter pylori* DNA was investigated using a bismuth-immobilized carbon nanotube electrode (BCNE). The analytical cyclic voltammetry (CV) peak potential was obtained at a 0.4 V reduction scan, where the diagnostic optimum square-wave (SW) stripping working range was achieved at 0.72–7.92 μg/mL *H. pylori* DNA (11 points). A relative standard deviation of 1.68% (RSD, n = 5) was obtained with 3.2 mg/mL *H. pylori* DNA using a 240 s accumulation time. Under optimum conditions, detection limit was 0.06 μg/mL. The developed sensors can be used for clinical application in the 15th doubted human gastric tissues, since the patient's peak current increased a hundred times more than the negative healthy tissue did. The sensing time obtained was only two minutes, and the process was simpler compared to common PCR amplification and electrophoresis photometric detection systems.

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1. Introduction

Helicobacter pylori and bacterial infection are the most important factors that affect the human-organ systems specifically involved in gastric carcinoma (Dulciene et al., 1998), gastric cancer (Antony et al., 1996), gastritis and peptic ulcer (Barrett et al., 1997), gastric lymphomas (Robert, 2001), duodenal ulcers (Kelly et al., 1999), and liver cancer (Philippe et al., 2000). *H. pylori* DNA and bacterial assay are particularly important for in-vivo or live cells and the blood vascular system. HP also resides in the saliva and in the stomach's internal-organ tissue, but it very rarely remains in the organ's fluid. Assays for related diseases demand very low analytical detection limits (DL) within molar ranges. At present, the most common analytical methods depend on PCR (polymerase chain reaction) DNA amplification and other analytical photometric and gravimetric detection systems that require devices such as self-assembled fluorescent nanosensors (Yina and David, 2007), resonant mirror biosensors (Momynaliev et al., 2003), and urinary test kits (RAPIRUN) based on the immunochromatographic method (Shunji et al., 1996). Enzyme-linked immunosorbent assay (Toru et al., 2001), capillary electrophoresis (Buszewski et al., 2007), the GC-MS detection of $^{13}CO_2$ enrichment in the ^{13}C urea breath test (Maraliese and Johannes, 2008), and the plasmid profiling of the amplified PCR method (Smith et al., 2002) have likewise been considered. Some of these techniques require an amplified PCR product (Bickley et al., 1993; Jang et al., 1999), gel electrophoresis separation, and fluorescence photometric-detection systems (Liviu et al., 2003; Agnes et al., 2001) and can be used only in qualified assays. Moreover, they are complicated, time-consuming and are not applicable in quantification analysis. Recently, simple and more sensitive voltammetric methods (Suw and Yun, 2006; German et al., 2007) have been developed, including screen-printed immunosensors (Monica et al., 2007) and electrochemical DNA biosensors (Suw, 2006), as well as the DNA hybridization detection method (Steichen et al., 2007). However, these have higher detection limits and are very complicated. For these reasons, better and more sensitive multiwall carbon nanotube (Suw, 2008) electrodes immobilized with catalytic bismuth were considered. In this aspect, the carbon nanotube structure is useful by virtue of its electrocatalytic property (Suw, 2005; Ge et al., 2004) and high electrical conductivity, and also because it promotes electron transfer. It also offers a large cylindrical surface area (Suw et al., 2007). Moreover, bismuth immobilization on a carbon nanotube is an attractive and viable replacement for toxic-mercury-composed electrodes in voltammetric sensors (Emily et al., 2006), since bismuth is environment-friendly and non-toxic (Minli et al., 2006; Lin et al., 2005). The Bismuth-CNE connected paste has large sorption capacity and surface pore increasing (Gadupudi et al., 2007) in the electrolyte solutions, which can produce supramolecular complexes for coordinated waters (Jane, 2003). Here, $[CNE\text{-}B(H_2O)_n]^{++}$ divalent complex metals react strongly with HP-DNA for $[CNE\text{-}B(H_2O)_n(HPDNA)_n]$. Under these conditions, the SW peak currents appeared to be very sensitive within the mole ranges using 240 s accumulation times. Here probe was successfully applied to the *H. pylori* DNA in gastritis

Abbreviations: BCNE, Bismuth immobilized carbon nanotube electrode; CV, Cyclic voltammetry; SW, Square wave stripping voltammetry; RSD, Relative standard deviation; CVD, Chemical vapor deposition.

* Corresponding author at: 172, gongreung 2-dong Nowon-gu, Seoul, South Korea 139-743. Tel.: +82 2 970 6691; fax: +82 2 973 9149.
E-mail address: suwyoung@snut.ac.kr (S.Y. Ly).

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and peptic-ulcer-disease patients, which can be usable for clinical gel electrophoresis, fluorescence photometric and diagnostic detection systems.

2. Material and methods

2.1. Systems

Analytical measurements were carried out using the Bioelectronics-1 system, which was pioneered by the authors' institution. Its new version is a computerized handheld voltammetric system with a 2.4 V potential range, a 2 mA current range, a 10 pico A measuring current, and having dimensions of 5″4″1″. It uses a rechargeable battery or external power and has a USB port interface with a PC. The instrument's size is similar to that of a typical cellular phone, and it can be used for bio assay and sensor techniques for individual and laboratory applications.

2.2. Reagents

The supporting electrolyte was prepared with a 0.1 M $NH_4H_2PO_4$ with pH 3.5 strength. All the other reagents were of analytical grade. Electrolyte voltammetry was carried out in an open circuit. A Bi standard solution (1000 mg L^{-1}) was obtained from Merck. Highly purified water was prepared through three-time distillation using 18 MΩ cm^{-1} of Milli-Q Ultra-Pure Water System (Millipore, Bedford, USA).

2.3. Electrode preparation

The BCNE was prepared by mixing 40% carbon nanotube graphite powder (multiwalled carbon nanotubes by catalytic CVD; outside diameter: 15×40 nm; length: 30×50 nm; Nanotech Co., Ltd., Choongnam, South Korea; these were purified overnight prior to use through magnetic stirring in a 2 M nitric-acid solution, then washed with triple-distilled pure water), 40% bismuth, and 20% mineral oil (New Jersey, USA, 1-800-01, Acro). Five grams of the mixture was homogenized in a mortar for 30 min. The 0.1 g paste was inserted into a needle-type capillary glass tube measuring 1.5 mm in diameter and 5 cm in length, and having a sensor connected to the electrochemical measurement system with a copper wire 0.5 mm in diameter. An Ag/AgCl electrode and a platinum wire served as the reference and auxiliary electrodes, respectively.

2.4. Procedures

The three-electrode system was immersed in a 10 mL 0.1 M $NH_4H_2PO_4$ solution. Under these conditions, BCNE immobilization was performed using a 10 cycle scan in a blank electrolyte solution, with a 1.0 V initial potential, a 1.0 V switching potential, and a 0.5 V s^{-1} scan rate, so that BCNE can be stabilized and then used in the optimization. All the experiments were performed at room temperature. Several pieces of the patient's gastric tissue were taken from the general hospital of the authors' institution. These tissue sample pieces were pooled and ground together. An aliquot of the sample homogenate was used for culture. The PCR DNA was isolated from 100 mL tissue using a Core One™ Tissue Genomic DNA isolation kit (www.corebio.com) with the manufacturer's guide Cat. No. TD 100. The DNA isolation procedure used was the proteinase K-lysis method. The DNA was eluted under a Tris buffer of 10-mM Tris–HCl and with pH 8.5, which could be used for voltammetric analysis. Under other gastric-tissue conditions, *H. pylori* DNA preparation and detection were performed using the common method, shown in Table 1 (Gadupudi et al., 2007; Jane, 2003; Bickley et al., 1993).

Table 1
PCR product conditions.

Target, nucleotide (nt) positions amplified, and sizes of the PCR products	Primer names and sequences	PCR conditions
glmM gene, nt784-1077, 294 bp	Forward primer: 5′-AAGCTTTTAGGGGTGTTAGGGGTTT-3′ Reverse primer: 5′-AAGCTTACTTTCTAACACTAACGC-3′	93 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min (35 cycles)

3. Results

3.1. CV Effects on H. pylori DNA

First, the cyclic peak potentials were examined using BCNE. Fig. 1 shows the positive *H. pylori* DNA variation using the patient's DNA. In the electrolyte blank solution, no redox peak current appeared as simple and linear. Thus, the 0.01 mL *H. pylori* DNA was spiked during the positive scan, and a 0.4 V peak potential was obtained. The reverse scan, however, did not obtain any signal. The *H. pylori* DNA was further spiked with the 0.02 and 0.03 mL, the oxidation currents exhibited a peak height that rapidly increased from 1.24×10^{-5} A to 1.86×10^{-5} A. Subsequently, the final peak, which appeared to be two times the peak height, was obtained. Thus, the method can be used in analyzing the patient's *H. pylori* DNA. Under these conditions, the common-type multiwalled carbon nanotube paste (non-bismuth-treated) electrode was compared with the same electrolyte conditions, and very weak signals were obtained. The BCNE peak was found to be much more sensitive than the common-type electrode. Based on the results, the peak potential can be applied to the diagnostic *H. pylori* infection, but more sensitive quantification methods were studied using SW stripping optimization, and were subsequently arrived at with more sensitive working ranges.

3.2. Analytical SW optimizations of BCNE

Under the 3.2 μg/mL *H. pylori* DNA spike, the SW stripping amplitude was varied at a nine-point range for 0–0.5 mV at a 30.0 s

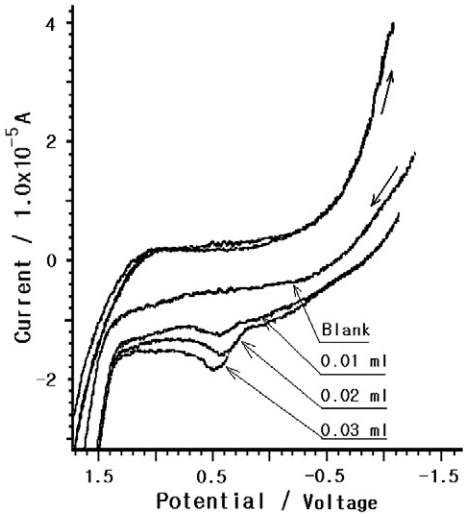

Fig. 1. Cyclic voltammetric concentration effects (reduction: →, Oxidation: ←) on *H. pylori* DNA. The first curve represents the electrolyte blank solution and the 0.01, 0.02 and 0.03 mL *H. pylori* DNA spikes (only ←) under the optimum conditions of −1.5 V initial potential, 1.6 V switching potential, and a 0.5 V s^{-1} scan rate, using BCNE and a 0.1 M $NH_4H_2PO_4$ electrolyte solution.

accumulation time, and the voltammetric peak current obtained is shown in Fig. 2a. The *H. pylori* DNA peak was shown to have increased slowly during the first five points, then very quickly during the next four points. It continued to rise from 1.445×10^{-6} A to 102.3×10^{-6} A. Subsequently, 0.025 mV was chosen as the optimum SW amplitude as its peak width was narrow and sensitive. Under this condition, the SW increment potential was examined using a seven-point variation, and the signal that was obtained from 4.38×10^{-6} A to 5.97×10^{-6} A is shown in Fig. 2a. The SW amplitude of 40 mV, which is high, was chosen. Under these conditions, the SW accumulation potential range of 0.0 to −2.0 V was varied, as shown in Fig. 2b, and its peak height was increased to 2.28×10^{-6} A~4.3×10^{-6} A. The optimum potential of −2.0 V is very sensitive, and as such, it was chosen for the adsorptive stripping voltammetry. The SW accumulation times were then examined under this condition. Fig. 2b shows the results of the voltammetric peak current that was conducted within the range of 0 to 240 s, using eight points. It continuously increased from 4.36×10^{-6} A to 5.76×10^{-6} A, and the 240 s accumulation time showed peak height results. The peak currents appeared to be more sensitive than the accumulation potentials, and they responded quickly. The peak width was likewise sharp. Thus, the 240 s accumulation time was used for all the other experiments. Under this condition, other parameters of SW frequency, SW increment potential, and electrolyte hydrogen ionic strength were optimized (data not shown). Finally, the optimum analytical SW conditions were set at 0.025 mV amplitude, 40 mV increment potential, −2.0 V accumulation potential, and 240 s accumulation time, while the other parameters including the pH 3.06 electrolyte hydrogen strength (In *H. pylori* patients with intragastric pH below 4.0: (Marek et al., 1996)) and the 30 mV SW frequency, were obtained (not shown here), appearing to be very sensitive and sharp. Thus, bismuth-composed BCNE is found to be suitable for analyzing HP DNA products. From here onwards, the analytical working ranges, diagnostic application, and statistics were examined.

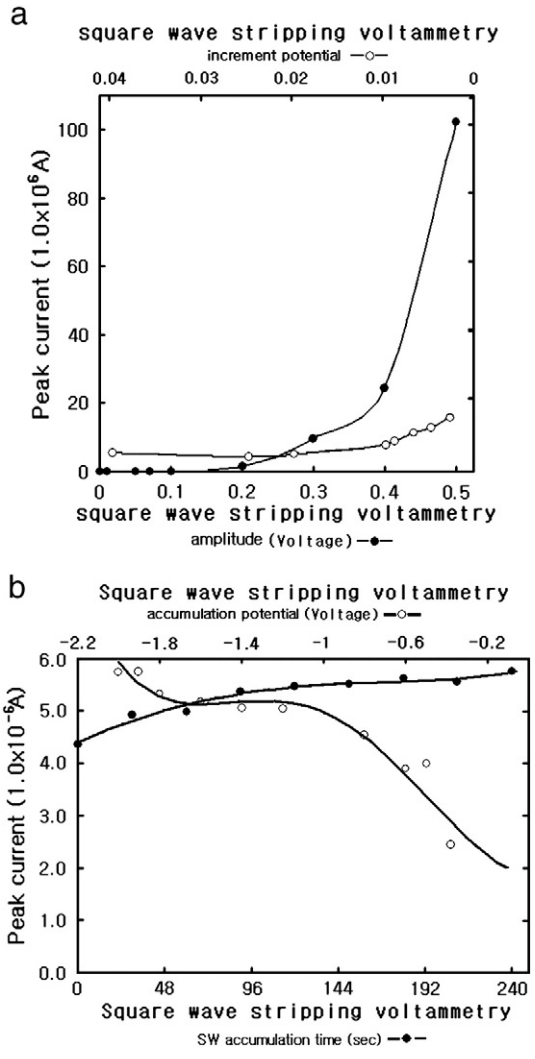

Fig. 2. (a) Variations of the SW amplitude, ranging from 0, 0.01, 0.05, 0.07, 0.1, 0.2, 0.3, 0.4, and 0.5 mV (—o—). SW increment potential range of 40, 25, 10, 9, 7, 5, and 3 mV (—●—). (b) SW accumulation potential range of 0, −0.4, 0.5, −0.6, −0.8, −1.2, −1.4, −1.6, −1.8, −1.9, and −2.0 V (—o—). SW accumulation times of 0, 30, 60, 90, 120, 150, 180, 210, and 240 s (—●—). The 3.2 μg/mL HP DNA spike in the 0.1 M $NH_4H_2PO_4$ electrolyte solution. Other parameters were used for the optimum conditions.

3.3. Working ranges and statistics

Using the optimized parameters, analytical linear working ranges were examined using a nine-point cyclic scan (not shown here). The blank electrolyte was simple, and no signal was observed with the 0.2 mL HP spike. More negative potential was obtained by the standard pH strength, and was continuously spiked from 0 to 5.76 mL. The linear working ranges appeared in the oxidation scan and were not obtained for the reduction currents. The slope sensitivity was $\triangle x/\triangle y = 333.2$, and peak currents of 11.81×10^{-6} A~62.2×10^{-6} A were obtained, indicating feasibility of use in HP DNA diagnosis. Then, an SW working range with a 12-point variation was examined using optimum conditions. Fig. 3 shows the real SW voltammograms. The peak width was very narrow and sharp, and it was used for the 240 s accumulation time. Since the slope ratio was $\triangle x/\triangle y = 220.06$ and the precision was $R_2 = 0.6338$ under the working condition, the methods were examined for their detection limits using KSb/m ($k = 3$, $n = 15$, $m = \triangle x/\triangle y$). The detection limit of 0.06 μg/mL (S/N = 3) SW was obtained, indicating that the SW method is much more sensitive than the CV method. Under these conditions, the statistical precision was examined using the replicated 15th determination of the 3.2 μg/mL HPDNA spike, from which 0.830% RSD was obtained. These results are highly reproducible and useful for HP diagnosis, making them suitable particularly for gastritis and peptic-ulcer disease.

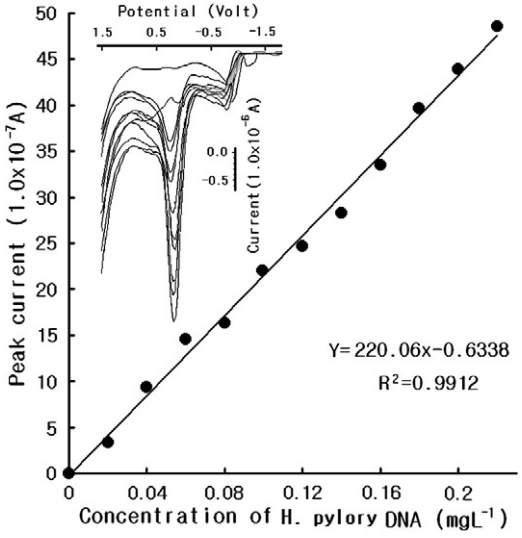

Fig. 3. SW working ranges of 0, 0.64, 1.28, 1.92, 2.56, 3.2, 3.84, 4.48, 5.12, 5.76, 6.4 and 7.04 μg/mL *H. pylori* DNA spikes in a 0.1 M $NH_4H_2PO_4$ electrolyte solution with a pH of 3.6, an SW amplitude of 0.025 mV, an SW frequency of 30 mV, an increment potential of 40 mV, an accumulation potential of −2.0 V, and an accumulation time of 240 s.

4. Discussion

4.1. Diagnosis for negative and patients DNA

The methods developed were used to measure the health of human DNA under optimum conditions. Absolute SW stripping voltammograms were examined using optimum parameters, although the signals were very simple and linear. Using this solution, the CV effects were examined, and the DNA peaks closely approached the blank signals. No current was observed when the following parameters were used: −2.0 V initial potential, 2.0 V switching potential, and 0.4 V/s scan rate. Under these conditions, statistical application was performed with 4th replication, but no current was obtained. Thus, the patient's positive-health HP DNA was examined under a newly prepared cell system. In the CV voltammograms with a blank solution, no signals were obtained. Then, 72 μg/mL *H. pylori* DNA from the patient was spiked, and a 0.54×10^{-5} A peak current with a positive direction was obtained, which was much larger than the blank noise (0.2×10^{-5} A). This indicates its feasibility for use in diagnosis. More sensitive SW voltammograms were thus examined using optimum parameters, and the results are shown in Fig. 4. The first curve represents the blank electrolyte, which is simple, and a 3.2 μg/mL *H. pylori* DNA spike appeared. This manifested with a peak current that has a sharp and narrow peak width of 0.35×10^{-5} and one hundred times the height of the blank electrolyte's noise (0.04×10^{-5} A). Thus, statistical analysis was carried out using the patient's healthy *H. pylori* DNA. Under optimum conditions, the previously analytically known 15th human DNA was examined. The statistical histograms were simple and were not shown here.

4.2. Statistical application

Fig. 5 indicates a level of negative-different health human DNA and the different patient's positive-DNA. In this DNA, the first SW is that of the positive *H. pylori* DNA, and peak currents ranging from 2.39 to 3.97×10^{-6} A were obtained, which were relatively high. Other patients' positive DNAs were examined using CV, and a peak current of $3.83\sim8.64\times10^{-6}$ A was obtained, which is more sensitive than the SW peak currents. This indicates feasible application in *H. pylori* DNA diagnosis. The final five points, however, were healthy DNA, whose peak currents were 0.03×10^{-6} A ~ 0.05×10^{-6} A. Negative-health DNA did not appear at any peak signal and noise level. The positive peak current increased one hundred times compared to the negative peak current. More exact comparisons were performed in a blind test using 15 known patients and common PCR methods, with all results coinciding with the previous results with nega = 0.06–0.09×10^{-6} A and posi = 2.3–3.2×10^{-6} A. The following optimum SW analytical

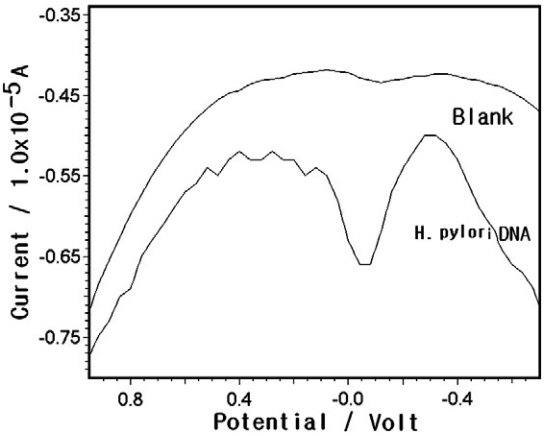

Fig. 4. Diagnostic applications for nega-blank and the patient's *H. pylori* DNA. Other conditions were used as optimum parameters.

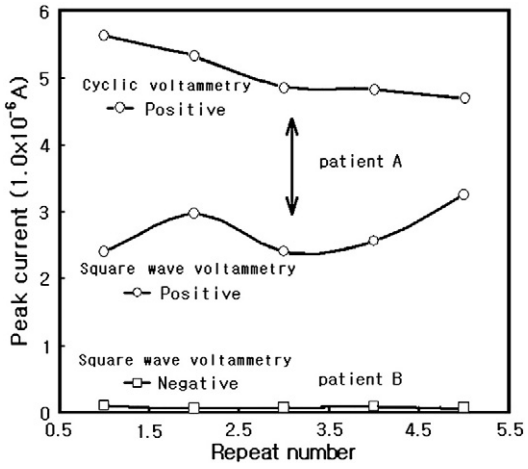

Fig. 5. Comparison of the effects due to the patient's negative and positive *H. pylori* DNA peak current using SW and CV voltammograms. The analytical optimum conditions in Fig. 3 were used.

conditions were used: 0.025 mV amplitude, 40 mV increment potential, −2.0 V accumulation potential, 0.1 M $NH_4H_2PO_4$ electrolyte solution, and analytical accumulation time of only 240 s. The diagnostic biosensor was successfully applied in detecting amounts of trace labels of HP DNA in gastritis and peptic-ulcer-disease patients. It can also be used in other fields requiring human DNA diagnostic analysis.

Potential conflicts of interest

None reported.

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