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Square-Wave Stripping Voltammetric Analysis of Human Papilloma Virus 58 DNA on Carbon Fiber Microelectrode

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Electrochemical behaviour of polymerase chain reaction (PCR) products of human papilloma virus (HPV) 58 DNA was studied employing cyclic voltammetry (CV) and square-wave stripping voltammetry (SWSV) on a carbon fiber micro-electrode (CFME) in order to develop a simple electroanalytical method to detect and determine HPV 58. This is to seek a possible substitute method for the common technique of gel electrophoresis and fluorescent image analysis for its detection. An anodic stripping peak that appears at -0.6 V (*versus* Ag/AgCl) was utilized for the determination. Optimum conditions for the square-wave adsorptive stripping analysis were found to be -1.0 V of deposition potential, 90 s of deposition time, 150 mV of SW amplitude, 4 mV of step potential and 120 Hz of SW frequency. A linear curve was obtained in a concentration range of 0.01-0.23 mg/L. The relative standard deviations at 0.01, 0.05 and 0.09 mg/L of concentration were 3.1, 2.7 and 5.3 % (n = 5), respectively at the optimum conditions. The detection limit (S/N) was found to be 0.006 mg/L. The method was applied to determine HPV 58 DNA from a real sample from a patient infected for the diagnostic purpose.

Key Words: Human papilloma virus 58, Square-wave stripping voltammetry, Cyclic voltammetry, Carbon fiber microelectrode, Polymerase chain reaction.

INTRODUCTION

Numerous reports have been appeared in the past[1-34] on the human papilloma viruses (HPVs) which belong to a group of small double-stranded DNA viruses consisting of *ca.* 8,000 base pairs. These epitheliotropic viruses are associated with both benign and malignant lesions and more than 80 different types of them have been found to date from DNA sequence analysis and other methods[20-22]. They can be classified into two major types[2,5,12,19,23]. Many of them, so-called high risk types such as HPV 16, 18, 31, 33, 35, 39, 45, 51, 56, 58, 59, are oncogenic causing cervical carcinoma[1,2,6-8,22,24], broncopulmonary[9] and other carcinomas[3,10,23]. On the

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other hand, the low risk types, such as HPV 6, 11, 42, 43, 44, 56, 61, 71, are benign. Thus, the diagnosis of HPV infection is a very important part of medical practices. During past few decades several assay methods to distinguish HPV-positive types from HPV-negative types have been developed[6-8,12-24] for cervical and other clinical samples. For general detection of all HPV genotypes, general or consensus primer-based polymerase chain reaction (PCR) assay methods have been developed[13-19]. In order to detect an infection of a particular genotype of HPV DNA, various schemes have been used including DNA sequence analysis[20], restriction enzyme analysis[21] and HPV type-specific hybridization of PCR products[19,22,27]. Most of these methods with polymerase chain reaction (PCR) involve follow-up experiments with agarose gel electrophoresis/fluorescence detection with ethidium bromide in order to analyze images (band patterns) developed which are specific for particular HPV genotypes. The analytical process of PCR products with this method of electrophoresis/fluorescence detection is very time-consuming and expensive. It normally takes hours to prepare the gels and apparatus and to separate products with agarose gel electrophoresis and to visualize the patterns with UV illumination and an image analyzer. Thus, the goal of this work is to search for a new, faster and simpler method for the detection of HPV DNA from PCR products for mass clinical samples with a different approach, namely employing electrochemical methods.

Electrochemical methods, voltammetric methods in particular, are generally simpler and faster than spectroscopic or any other analytical methods[35,36]. Thus, in order to enhance electrochemical current signals, we first amplified concentrations of the HPV 58 specific DNA oligomers through a PCR reaction, then studied the PCR products from the HPV 58 DNA using cyclic voltammetry (CV) and square-wave voltammetry (SWV) in a stripping mode[36]. Stripping voltammetry has been very popular in recent years because of its speed and sensitivity[36]. There are a number of reports on the detection of DNA and DNA sensors with electrochemical methods including several review articles[37-39]. The examples of application includes the detection of hepatitis B virus[40] with hybridization indicator methylene blue[41], of factor V Leiden mutation[42], of the DNA of a Chinese medicinal herb[43], of apolipoprotein E genotypes[44], of the HIV virus[45] and others[46-49]. Many of these methods involve the immobilization of particular oligonucleotides, which is complementary to a target DNA and the subsequent hybridization which leads to the binding of various electroactive indicators (such as metal complexes[45] and intercalators or groove binders[43]) in order to generate current signals from a redox reaction. On the other hand, methods that depend on the intrinsic redox signals of DNA themselves, *i.e.*, without any indicators, have also been developed[50-53] because DNA itself is known to be electroactive[38] - the guanine and adenine moiety are susceptible to redox reaction. Such direct methods that can bypass the hybridization steps appear to be more attractive. Additonal work on the various electrochemical sensing of DNA can be found elsewhere[54-58]. Thus, in the present work, we produced particular DNA oligomers (HPV 58 specific) from PCR reactions and tested its electrochemical

properties and searched for conditions that can best produce stripping currents for the direct determination of HPV 58 DNA with a square-wave anodic stripping voltammetry on carbon fiber microelectrode (CFME)[35]; CMFE is suitable for a small volume analysis and miniaturization. Optimum values of pH, deposition (accumulation) potential, deposition time, square wave (SW) amplitude, square wave frequency are reported for the determination.

EXPERIMENTAL

Cyclic voltammetry and square wave stripping voltammetry measurements were performed using the CHI 660A electrochemical workstation (CH Instruments Inc, Cordova TN, USA). A three-electrode system was used to record the voltammograms. The working electrode was a carbon fiber micro electrode (CFME) of 4 μm diameter. The reference electrode was a Ag/AgCl electrode (saturated KCl) and the auxiliary electrode was a platinum wire.

Preparation of polymerase chain reaction (PCR) products of HPV 58 DNA: As suggested in the work of Husnjak *et al.*[15], we used two sets of consensus primers, MY09/MY11 and GP5/GP6[15,16]. The conditions of the amplification of HPV 58 DNA with the first PCR reaction for 450 base pairs are following. The PCR reaction mixtures consist of a template of HPV 58 DNA and degenerate primers (MY09/MY11) for the conserved PCR for HPV[14-19]: 5' primer (forward, MY11): 5'-GCM CAG GGW CAT AAY AAT GG-3'; 3' primer (reverse, MY09): 5'-CGT CCM ARR GGA WAC TGA TC-3'.

The PCR buffer was made of 0.75M *Tris*-Cl (pH 9.0), 0.15 M $(NH_4)_2SO_4$, 1 mg/mL BSA, 0.025 M $MgCl_2$, Taq DNA polymerase, dNTP (dATP, dTTP, dGTP, dCTP), distilled water.

The oligonucleotides primers were either synthesized in the laboratory with DNA Synthesizer Vers. 03 (Polygen, Germany) or purchased from Metabion (Germany) and MWG (Germany). The PCR reactions were carried out with 40 cycles of amplification using Thermocycler 9700 (ABI Biosystems, USA) after a first step of denaturation at 95 ºC for 5 min. Each cycle includes a denaturation step at 95 ºC for 30 s, a primer annealing step at 40 ºC for 30 s and a chain elongation step at 72 ºC for 1 min. The second amplification was done using another set of primers (GP5/GP6) at the same conditions, except with 20 cycles of amplification. The base sequences of primers were: 5' primer (forward, GP5): 5'-TTT GTT ACT GTG GTA GAT ACT AC-3'; 3' primer (reverse, GP6): 5'-GAA AAA TAA ACT GTA AAT CAT ATT C-3'.

Preparation of working electrode and procedure for electrochemical measurements: The carbon fiber microelectrode has a dimension of 4 μm of diameter and is 15 mm in length. The electrode was attached to a copper wire *via* silver paint, then the fiber was inserted into a polyethylene tube of 0.3 mm diameter. The electrode was sealed by heating and was cleaned by sonication for 3 min first in acetone, then in nitric acid (1:1) and finally in double-distilled water. All solutions

were prepared from triply-distilled water (18 Moh cm^{-1}). Other standard stock solutions were obtained from Aldrich and diluted as needed. The 0.1 M phosphate buffer solution served as a supporting electrolyte. Three electrode system was immersed into a 15 mL cell which contains 10 mL of 0.1 M Na_3PO_4 and 0.1 M $(NH_4)_3PO_4$ buffer solution (pH 6.0). The pH of the buffer was adjusted with either 0.1 M HCl or 0.1 M NaOH to various values as needed. PCR products of the HPV 58 DNA solutions of 0.20 mg/L was spiked into the buffer solution for the electrochemical measurements. Cleaning of electrodes was not necessary between measurements.

RESULTS AND DISCUSSION

Before assessing optimum experimental conditions for a sensitive determination of HPV58 DNA with the square-wave stripping voltammetry, some general electrochemical behaviours of HPV 58 were investigated first with the common cyclic voltammetric method[35].

Cyclic voltammetry and effect of pH: Fig. 1(A) shows cyclic voltammograms of 0.20 mg/L HPV 58 solution at various pH values obtained at a scan rate of 130 mV/s. pH of the phosphate buffer was adjusted using hydrochloric acid and sodium hydroxide. At pH 4, a cathodic peak appeared at about -0.68 V; then the peak potential shifts to the positive direction as pH increases with a value of -0.43 V at pH 7. This suggests that H^+ is taken up in the reduction process. The cathodic peak is believed to be a reduction of an oxidized guanine moiety because the starting potential was sufficiently positive for an oxidation of guanine[50,51]. The anodic peak at 0.40 V at pH 4, formed from the oxidation of guanine, behaved in a similar fashion yielding a peak potential at 0.59 V at pH 7. The oxidation is a two-step mechanism involving a total loss of 4 e^- and 4 H^+ from guanine[50]. Linear regression of the anodic peak potentials *vs.* pH yielded an equation (y = 59.4x + 154). The observed slope of 59.4 mV agrees very well with the theoretical value of 59.0 mV to support the mechanism of 4 e^-/4 H^+ reduction process.

In Fig. 1(B), the peak currents as a function of pH are plotted. As the pH value increases from 4, both cathodic and anodic currents increases reaching maximums at a pH value of 6, then they (the absolute values) decrease. Thus we selected this pH value, 6, for other square-wave voltammetric measurements.

Cyclic voltammetry peak current at various HPV 58 concentrations: In Fig. 2, peak currents at various concentration of HPV 58 solutions are plotted and results from regression analysis is also given. Both anodic and cathodic peaks yielded straight lines in a range of 0-0.4 mg/L. The anodic currents gave an equation of y = 0.2107 x and the cathodic current gave y = 0.1075 x with correlation coefficient of 0.9551 and 0.8848 respectively (y = current, pA; x = HPV 58 concentration, mg/L). Since the reduction currents are twice as sensitive as the oxidation currents, we choose the reduction peak, rather than the oxidation peak for the square-wave stripping analysis.

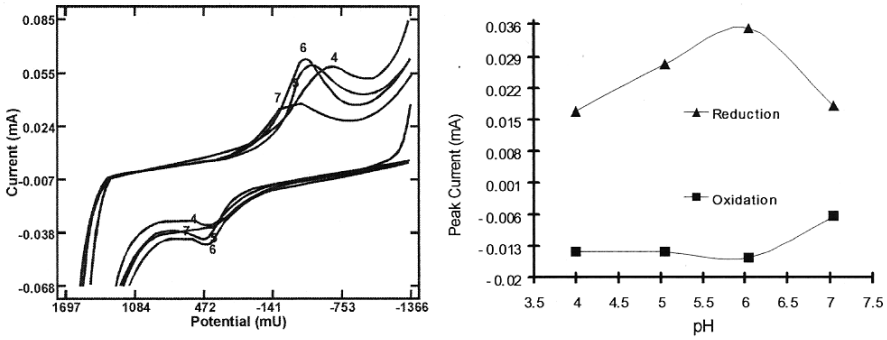


Fig. 1. (A) Cyclic voltammograms of 0.20 mg/L HPV 58 solution at various pH; initial potential: 2.0 V, switching potential: = -1.50 V, scan rate: 130 mV/s. (B) The peak currents at various pH values

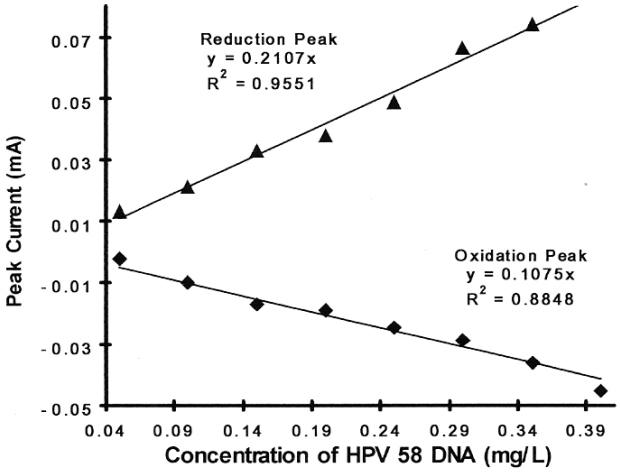


Fig. 2. Cyclic voltammetric anodic and cathodic peak currents at various concentrations (0.1-0.4) mg/L HPV 58 in the 0.1M phosphate buffer with the carbon fiber micro electrode

Square-wave anodic stripping voltammetry: In this section, we searched for optimum conditions for the SW anodic stripping voltammetry that can yield the highest current signals for the HPV 58 solutions.

Effect of accumulation potential and accumulation time on the stripping peak current: Examination of the influence of the various pre-concentration potential was carried out over the range -0.8 to -1.9 V. The dependence of the stripping peak current on the deposition potential of HPV 58 in the phosphate buffer solution is shown in the Fig. 3(A). A maximum current of the HPV 58 DNA is obtained at

about -1.0 V. Therefore, we chose this accumulation potential in the adsorptive stripping voltammetric determination of HPV 58 DNA.

Fig. 3(B) demonstrates the dependence of the stripping voltammetric peak currents on the accumulation times in a range of 15-240 s. The stripping conditions of the electrodes system were an initial potential of -1.1 V, a final potential of 0.2 V and a quiet time of 2 s. Other SW parameters used were a frequency of 120 Hz, a step potential of 4 mV, an amplitude of 0.125 V. Peak currents increased rapidly to about 60 s, after which the increase diminishes substantially. A time of 60 s appears to be long enough in reaching the maximum adsorption for different values of HPV concentration as well. Thus, the optimum accumulation time was chosen as 60 s for the stripping analysis of HPV 58 DNA.

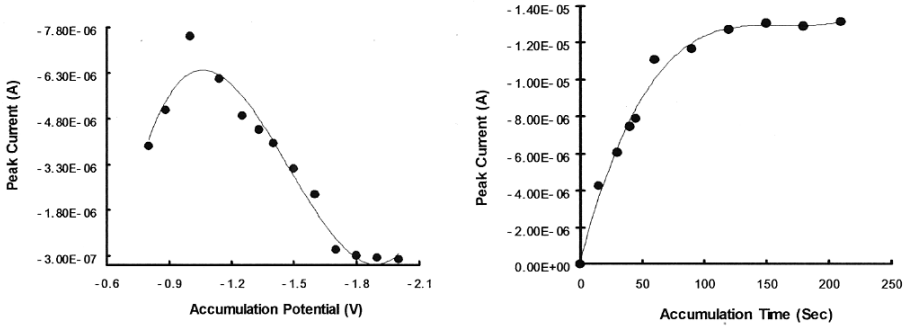


Fig. 3. (A) Effect of deposition potentials (-0.8 to 1.9 V) on the stripping peak current. Conditions: 0.2 mg/mL of HPV 58, deposition time of 60 s, square-wave frequency of 120 Hz, a square-wave amplitude of 125 mV, a step size of 4 mV; (B) Effect of deposition times (15, 30, 40, 45, 60, 90, 120, 150, 180, 210, 240 s) on the stripping peak current. Deposition potential of 1.0 V, other conditions are the same as in Fig. 3 (A)

Effect of the SW parameters (the amplitude, frequency and step potential) on the stripping currents: Fig. 4(A) illustrates the stripping peak current of 0.2 mg/L HPV58 DNA solution as a function of a square-wave amplitude with a pre-concentration time of 60 s. Other experimental parameters were kept the same as in Fig. 3. The range of the amplitude studied was in between 0.001 and 0.2 V. As one can expect the peak current increases as the amplitude increases. It increases rapidly at low amplitudes, then it starts to level off at around 0.125 V. Thus, 0.125 V was chosen as the optimum pulse height. The dependence of the peak current on the square-wave frequency is shown in Fig. 4(B) in a range of 5-120 Hz, where other experimental parameters were kept the same as in Fig. 3. At the beginning, current increases upto about 30 Hz and it starts to level off (or decrease) in a range of 50-80 Hz. Then it starts to increase again at about 80 Hz. A frequency of 120 Hz appears to be an optimal value.

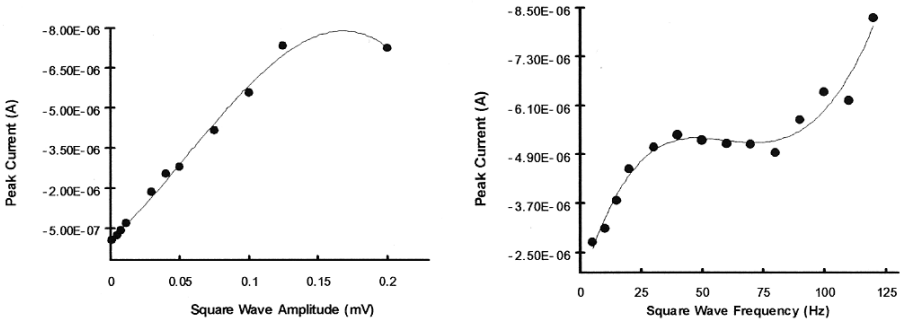
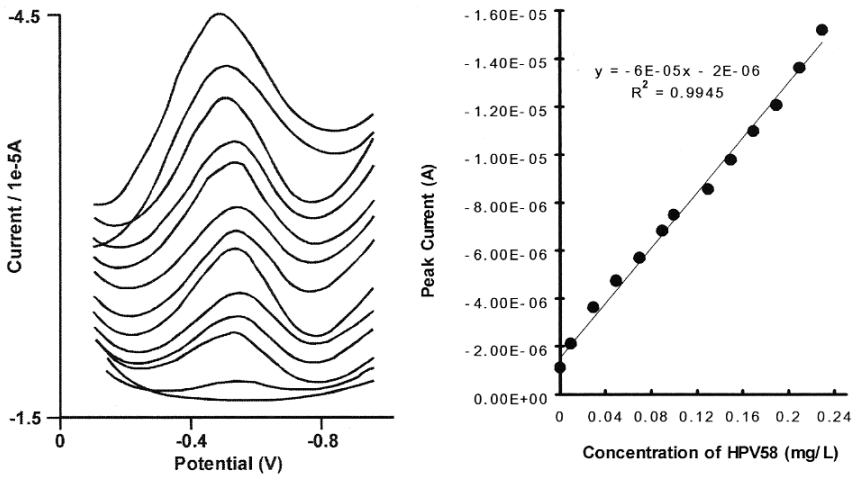


Fig. 4. (A) SW stripping peak currents as a function of SW amplitude for 0.20 mg/L. HPV 58 solution. Other conditions are the same as in Fig. 3; (B) SW stripping peak currents as a function of SW frequency, other conditions are the same as in Fig. 3

Effect of concentration of HPV 58 DNA, a linear range and detection limits: Fig. 5(A) shows raw SW voltammograms of HPV58 DNA at various concentrations (0.00, 0.01, 0.03, 0.05, 0.07, 0.09, 0.1, 0.13, 0.15, 0.17, 0.19, 0.21, 0.23 mg/L) at the optimum conditions. The peak currents are plotted as a function of a concentration in Fig. 5(B) with a result from a regression analysis. The stripping peak currents gave a linear equation of y = - 60x - 2 with correlation coefficient of 0.9945 (12 points; y = current, μA; x = HPV 58 concentration, mg/L). The precision for 12 successive determinations of 0.07 mg/L with a 60 s accumulation time was 0.02 % (RSD), the detection limit was estimated to be 0.006 mg/l (SN = 3) at the optimum conditions.

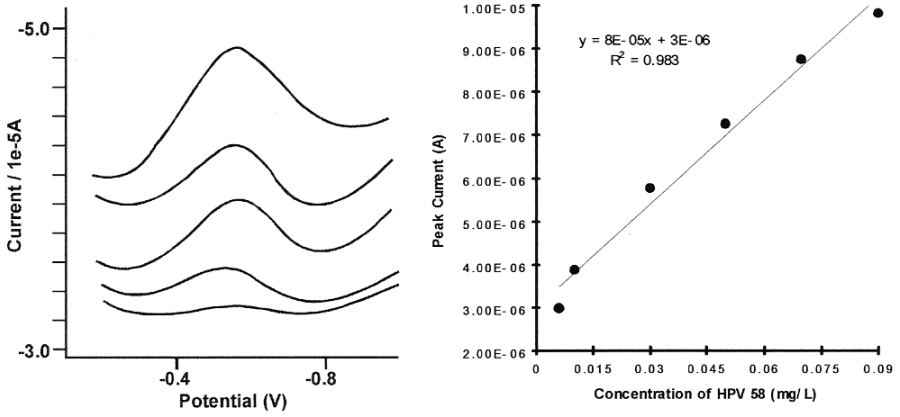
We tested the SW stripping method with the optimum conditions in order to detect HPV 58 DNA from PCR products of real female cervical samples. A positive sample means that it is infected with HPV 58, a negative sample means that it is not.

Fig. 6(A) shows raw stripping voltammetric curves of PCR product from a real human female samples which is infected with HPV 58. The curves are obtained at different amounts of the PCR sample added to the buffer with concentrations of 0.01, 0.03, 0.05, 0.07, 0.09 mg/L. The peak height increases as the concentration increases. In Fig. 6(B), the peak currents are plotted as a function of a concentration; a base current of the background are also given which is the lowest one. This clearly demonstrates that the present stripping methods can be used not only for a detection of HPV 58 infection but also for a quantitative analysis of it. This is an important advantage for a HPV nucleic acid analysis because it can bypass the time-consuming method of electrophoresis/fluorescence detection. The concentration of HPV 58 in the sample was determined by the standard addition method with known concentrations. Five negative PCR samples that does not have HPV 58 did not produce any stripping peak around -0.6 V, while only positive PCR samples products, which are infected with HPV 58 virus, yielded the oxidation peak.



(A) (B)

Fig. 5. (A) Square wave stripping voltammograms at various concentrations of HPV 58 DNA (0.0, 0.01, 0.03, 0.05, 0.07, 0.09, 0.1, 0.13, 0.15, 0.17, 0.19, 0.21, 0.23 mg/L) on a carbon fiber microelectrode. Deposition potential, -1.0 V; deposition time, 60 s; initial potential, -1.0 V; final potential, 0.1 V; SW amplitude, 120 mV, SW frequency, 120 Hz; SW step height, 4 mV; (B) The peak current as a function of concentration



(A) (B)

Fig. 6. (A) Square wave stripping voltammograms of a positive sample spiked into the buffer at various amounts. Concentrations from the bottom: 0.01, 0.03, 0.05, 0.07, 0.09 mg/L. The conditions were the same as in Fig. 6; (B) The peak current as a function of concentration for the background electrolyte and the solutions of the positive sample

Conclusion

A simple method for detection and quantitation of HPV 58 DNA from PCR product was demonstrated using square-wave stripping voltammetric measurement at a carbon fiber microelectrode. This was accomplished by utilizing the voltammetric reduction peak at around -0.6 V. Optimum analytical conditions for the stripping method were determined. The electrode response was linear to the HPV 58 DNA concentration within the range of 0.01 to 0.23 mg/L, with a detection limit of 0.006 mg/L (S/N = 3) with 60 s of accumulation time. The coefficient of variation at 0.01, 0.05 and 0.09 mg/L of concentrations were 3.1, 2.7 and 5.3 % (n = 5), respectively, at the optimum conditions. The detection limit (S/N) was found to be 0.006 mg/L. The method was applied to PCR products from real DNA samples of squamous cervical cancer cell. The method is much simpler and faster than the common electrophoresis/fluorescence detection method with the image analysis of band patterns.

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