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Electrochemistry

A SENSITIVE ELECTROCHEMICAL ASSAY FOR EARLY DETECTION OF HIV-1 PROTEASE USING FERROCENE-PEPTIDE CONJUGATE/AU NANOPARTICLE/SINGLE WALLED CARBON NANOTUBE MODIFIED ELECTRODE

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A rapid and simple electrochemical detection method has been developed to detect the presence of protease (HIV-1 PR) in HIV patients' sera. In this assay format, disposable screen printed gold electrode (SPGE) surface was modified with gold nanoparticles (AuNPs) or thiolated single-walled carbon nanotubes|gold nanoparticles (SWCNT| AuNPs). Thiol-terminated ferrocene (Fc)-pepstatin was then self-assembled on such modified surfaces. The interaction between the Fc-pepstatin modified substrates and HIV-1 PR was confirmed by scanning electron (SEM) and atomic force microscopy (AFM) and probed by differential pulse voltammetry (DPV). A linear relationship was observed between the shift in peak potential and the increasing number of HIV-1 viral replications in the HIV-infected serum samples.

Keywords: Electrochemistry; Ferrocene; Gold nanoparticle; HIV-1; Pepstatin; Protease; Single-walled carbon nanotube

INTRODUCTION

The sensitivity of assays for detecting low levels of Human Immunodeficiency Virus 1 (HIV-1) antibody has become an important issue as far as early diagnosis is concerned. A specific antibody to HIV is produced shortly after infection, but the exact time depends upon several factors including host and viral characteristics. Importantly, the antibody may be present at low levels during early infection which is beyond the detection limit of some assays (Parekh and McDougal 2001). The early-generation tests could detect the antibody in most individuals

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by 6 to 12 weeks after infection. Newer-generation assays, including the third-generation antigen sandwich assay, can detect antibodies at about 3–4 weeks after infection. To date, HIV-DNA PCR is the most widely used initial assay for early diagnosis in developed countries. Besides accuracy and reliability, HIV-DNA PCR and other nucleic acid detection techniques provide important information about virological status that can assist in clinical management (Marie Louie and Simor 2000; Elfath et al. 2000; Ballagi-Pordany 1993; Niesters 2004; Hjelle and Busch 1989). However, both HIV-DNA and HIV-RNA require specific equipment and consumables that are often not available and/or affordable in developing countries (http://www.who.int/hiv/en). Therefore, there is a critical need for affordable alternatives.

Aspartic protease is one of the three key enzymes of HIV-1 virus. This 10–12 kDa protein is required for the post-translational cleavage of the precursor polyproteins, Pr_{gag} and $Pr_{gag\text{-}pol}$. These cleavages are essential for the maturation of HIV infectious particles (Kohl et al. 1988; McQuade et al. 1990; Seelmeier et al. 1988). Thus, the protease enzyme becomes one of the key targets for developing anti-AIDS drugs and/or the early detection assay for HIV virus. A variety of biosensing platforms and detection strategies has been developed as reliable tools for the recognition of HIV virus (Iqbal et al. 2000; Taber, Couch, and Dominguez 1993; Lennette and Smith 1999). Portable and rapid biosensing platforms offer a promising alternative to existing analytical methods especially in geographical regions where medical facilities are deficient (Amano and Cheng 2005; Pejcic, De Marco, and Parkinson 2006).

Recently, we have demonstrated an ultrasensitive electrochemical method for the detection of HIV-1 PR with a detection limit of 0.8 pM (Mahmoud, Hrapovic, and Luong 2008) and a reliable AID drug screening assay based on electrochemical impedance (Mahmoud and Luong 2008). This paper describes a sensitive electrochemical assay for the detection of HIV-1 PR that is more amenable to applications in the field and array format. The procedure is engineered by modifying a disposable screen printed gold electrode (SPGE) with AuNPs-SWCNT to attain reliable detection sensitivity to protease in the HIV-infected blood samples. The electrode surface is modified with thiolated single walled carbon nanotubes (SWCNT)/gold nanoparticles (AuNPs). Thiol-terminated Fc-pepstatin is then self-assembled on such surfaces as the sensing probe. The interaction and binding between the Fc-pepstatin-modified substrates and HIV-1 PR, that is adequately present in HIV-infected serum is studied by scanning electron microscopy (SEM), atomic force microscopy (AFM), and differential pulse voltammetry (DPV).

EXPRIMENTAL

Reagents and Materials

The HIV-1 PR recombinant expressed in *Escherichia coli* (25 μg), human serum albumin (HSA) and human male serum were purchased from Sigma (St Louis, MO). DTT (±threo-2,3-dihydroxy-1,4-butanedithiol) was obtained from Fluka. Ferrocene (Fc)-conjugated pepstatin (Cys-(NH-Pro_4-C(O)-Fc-C(O)-Val_2-Sta-Ala-Sta-OH)$_2$

was prepared as described elsewhere (Kerman, Mahmoud, and Kraatz 2007; Xu and Kraatz 2001). Single walled carbon nanotubes (SWCNT) (diameter of 1 nm, purity >90 wt%, length: 5–30 μm, specific surface area: 407 m^2/g, electrical conductivity: $>10^{-2}$ S/cm) were purchased from Carbon Nanotechnology (Houston, TX). The screen-printed gold electrodes (SPGE) were kindly donated by Prof. Eiichi Tamiya (University of Osaka, Japan) and Biodevice Technology Ltd (Ishikawa, Japan). The total length of the strip was 11 mm with a geometric working area of 2.64 mm^2. All other chemicals were obtained from Sigma-Aldrich (St Louis, MO) and used as received.

SWCNT/AuNP Modified Gold Electrode

The pretreated SWCNT (1 mg) was dispersed into 2 mL absolute ethanol and sonicated for 2 h to generate a black suspension. A SPGE was coated with 10 μL SWCNT and allowed to dry under an argon stream. After thorough rinsing, gold nanoparticles were deposited electrochemically onto the SWCNT modified electrode by following the same procedure as described previously. The peptide films were immobilized on SWCNT/AuNP electrodes as described by Mahmoud and Luong (2008). In brief, the electrodes were immersed in ~1 mM thiol-terminated Fc-pepstatin conjugate prepared in 5% (by volume) acetic acid in ethanol for 12 h. The HIV-1 PR stock solution (20 nM, prepared in 0.1 M sodium acetate, pH 4.7, containing 2 mM EDTA, 1 mM DTT, and 10% DMSO) was incubated at 23°C for 1 h prior to the measurement. The activated enzyme was kept on ice. Subsequent dilution of the enzyme was prepared by using the assay buffer. The peptide modified electrodes were incubated with different HIV-1 PR concentrations for 1 h and washed twice with the assay buffer and deionized water. Control experiments were carried out with 75 μM HSA with and without HIV-1 PR.

Serum Sample Measurement of HIV-Infected Patients

Serum or plasma specimens with a known viral replication count (copies/ml) as well as unknown samples were collected in 7-mL EDTA vacutainers and stored at −20°C. The stock plasma solution was diluted in the HIV-1 PR activation buffer to a final volume of 0.8 mL. For electrochemical measurements, 100 μL of a buffered sample was spotted on the SWCNT/AuNP/Fc-pepstatin modified screen printed electrode and differential pulse voltammetry (DPV) measurements were performed in a pH 7.4 buffer medium.

Instrumentation

Cyclic voltammetry (CV), DPV, and amperometric measurements were performed using an electrochemical analyzer coupled with a picoamp booster and a Faraday cage (CHI 760B, CH Instruments, Austin, TX). A platinum wire (Aldrich, 99.9% purity, 1 mm diameter) and an Ag/AgCl, 3 M NaCl (BAS, West Lafayette, IN) electrode were used as counter and reference electrodes, respectively. The SPGE

and conventional gold disk electrodes were polished with polishing paper (grid 2000) and, subsequently, with alumina until a mirror finish was obtained. The electrodes were sonicated for 5 min to remove the alumina residues followed by thorough rinsing with water and ethanol. The electrodes were conditioned by cyclic voltammetry between 0 and +1.4 V vs. Ag/AgCl in 0.5 M H_2SO_4 at 100 mVs^{-1} until a stable CV profile was obtained.

The AFM measurements were carried out by a Nanoscope IV system (Digital Instruments-Veeco, Santa Barbara, CA), operating in air in tapping mode. Silicon AFM probes with a cantilever length of 125 mm and drive frequencies of 235–255 kHz were employed for imaging. The image scan speed was 0.50 Hz at 512 lines/scan. All height measurements and size distributions were obtained using the software package of the instrument. SEM (Hitachi, S-2600 N, Tokyo, Japan) operating in high vacuum mode with an acceleration voltage of 10–24 kV and a working distance of 3–15 mm, depending on the sample was used to analyze the morphology of SWCNT before and after different modification steps.

RESULTS AND DISCUSSION

For the development of an efficient detection method for HIV virus in blood at early infection, it is logical to target a viral component that is adequately present even at very low viral replications. The HIV Protease (HIV-PR) is one potential candidate. We anticipate an interaction and binding between the Fc-pepstatin-modified substrates and HIV-1 PR that is present at enough concentration in HIV-infected serum samples. The study was assessed using SEM, AFM, and DPV.

The formation of a stable monolayer of Fc-pepstatin conjugate on SWCNT/AuNP modified SPGE has been described in detail elsewhere. In brief, a thin film of pre-thiolated SWCNT was coated on the electrode surface followed by the formation of AuNPs on the thiol terminals by electrochemical deposition from a 0.5 M H_2SO_4 solution containing 5 mM $HAuCl_4$. The monolayer of cystamine containing the Fc-pepstatin conjugate was formed on the SWCNT/AuNP modified electrode surface through well-known Au-S interactions.

The formation of the SWCNT/AuNP composite was confirmed by SEM and AFM as shown in Figures 1 and 2, respectively. From SEM, granular clusters of AuNPs of the SPGE (Figure 1a) can be clearly seen with an average diameter of 1–3 μm. As shown in Figure 1b, the electrode surface was covered by a denser layer after the deposition of AuNP/SWCNT. Notice also that Fc-pepstatin effected a significant change of the electrode morphology (data not shown). Upon the incubation of modified SPGE with in a buffer containing 10 nM HIV-1 serum samples, the electrode surface was subject to an additional stress, resulting in significant alteration of the surface morphology (Figure 1c).

The AFM was used to probe the morphological change of the electrode surface during the course of measurement. After the interaction with HIV-1 PR, the electrode surface became more uniform due to the distribution of the protein clusters that were positioned above the SWCNT/AuNP/Fc-pepstatin film (Figure 2). The well-shaped surface morphology could be attributed to various protein orientations or the formation of a protein multilayer on the background surface, indicative of

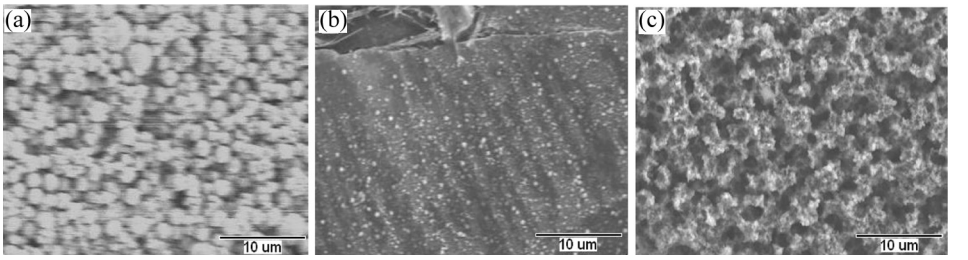

Figure 1. SEM images of: (a) a bare screen printed gold electrode (SPGE) (5000 ×); (b) AuNP/SWCNT modified SPGE (6000 ×); and (c) HIV-1 PR/AuNP/SWCNT (4500 ×). The granular AuNPs of the SPGE: (a) were covered by a higher dense layer after the deposition of AuNPs/SWCNT; (b) upon incubation with HIV-1 serum samples; and (c) an additional stress influenced the morphology of the electrode surface.

HIV-1 PR interaction with the Fc-pepstatin film. The resulting modified electrode material allowed for extremely low detection sensitivity for HIV-1 PR and improved the electrode's response to the binding event due to its higher exposed surface area provided by the SWCNT network.

The process electrode preparation and surface recognition of HIV-1 PR was first confirmed by DPV as shown in Figure 3. The position of the Fc group in close proximity to the electrode surface allowed for monitoring the changes in the electrode potential before and after the interaction with increasing concentration of the enzyme. The films displayed an oxidation peak at $E_{ox} = 0.437 \pm 0.005$ V at 0.1 V/s vs. Ag/AgCl. Upon the selective binding of HIV-1 PR to the surface bound inhibitory peptide (Fc-pepstatin), a shift to higher potential together with a decrease in the peak current intensity was observed. This change could be attributed to blocking of the penetration of the supporting electrolyte to the electrode surface, thereby decreasing its ability to oxidize Fc. Above 5 pM enzyme concentration (Figure 3,

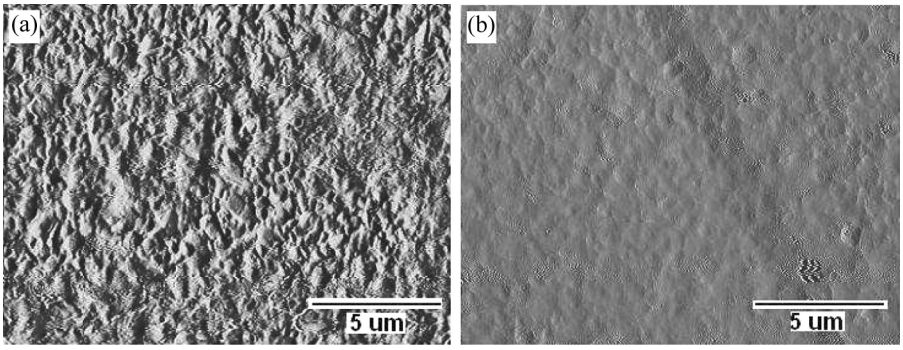

Figure 2. AFM micrographs of the SWCNT/AuNP/Fc-pepstatin film on SPGE (a) before and (b) after incubation with 10 pM of HIV-1 PR. The micrograph was taken in situ for the dried samples after successive washing with the buffer solution and deionized water. The figure shows the change of the pattern from rough surface to uniform protein aggregates, which are positioned above the peptide modified surface as evident from AFM imaging.

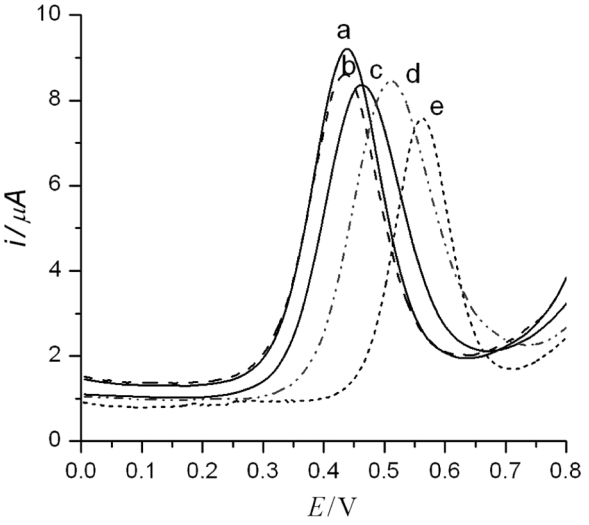

Figure 3. DPV of SWCNT/AuNP modified gold electrodes modified with Fc-pepstatin conjugate in the presence of different concentrations of HIV-1 PR at: (a) 0 pM, (b) 5 pM, (c) 10 pM, (d) 100 pM, and (e) 1000 pM. Ag/AgCl was used as the reference electrode at 100 mV/s. The assay buffer consisted of 0.1 M sodium acetate, 2 M $NaClO_4$, 1 mM EDTA, 1 mM DTT, 10% DMSO, pH 7.4. The E° of the Fc/Fc^+ couple under the experimental conditions is 448 (±5) mV.

curves c–e), a well-pronounced shift in the formal potential ($\Delta E = 55$ mV) was observed.

The real samples assay was designed based on the electrochemical detection of HIV-1 protease in blood samples with different HIV-1 viral replication stages and was carried out by using a disposable SPGE. Similarly, the recognition layer was assembled on the electrode surface as described previously. The changes of the Fc-pepstatin oxidation signal were also monitored electrochemically in the same DPV scale using the HIV-PR activation buffer and in the presence of the HIV-1 infected blood plasma. Figure 4a shows the oxidation signal of Fc-pepstatin at $E_{ox} = 0.431 \pm 0.007$ V. In the presence of non-infected blood serum (control), only a decrease in the current was observed without a noticeable shift in the peak potential. However, when HIV-infected serum samples with increasing viral replications were introduced, a positive shift of the peak potential was observed. This resulted in a linear correlation between the shift in the peak potential and the number of the viral replicates as illustrated from Figure 4b. Each measurement was repeated three times to obtain the mean and the corresponding error bars. The selectivity of Fc-pepstatin binding is clearly observed. While there was no response to the control blood serum, a strong potential shift of 98 mV was recorded in the presence of small viral amount (680 copies/mL). The peak shift (164 mV) was obtained at the highest viral replications of 1.14×10^6 copies/mL. Notice that the change of the peak current is irrelevant to the viral concentration and most likely is due to the cholesterol density in the blood samples from different HIV-infected patients (lipodystrophy) as confirmed by the blood test.

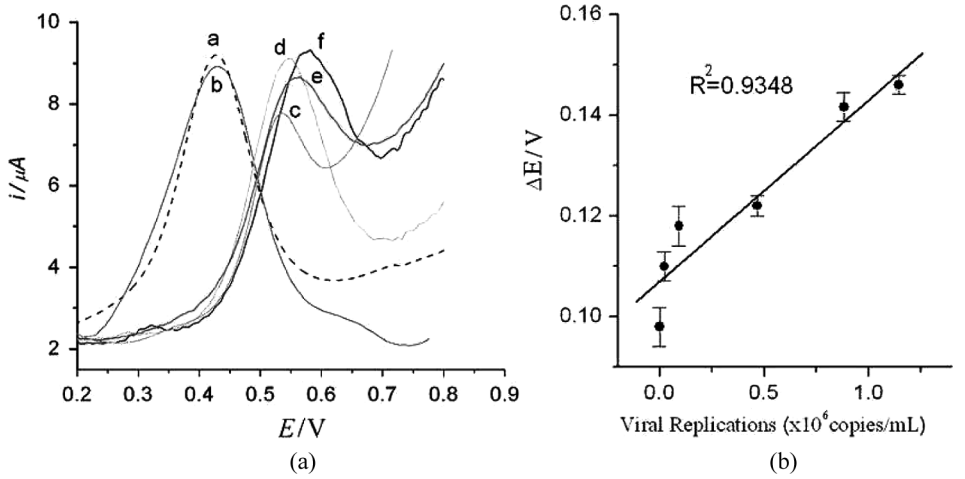

Figure 4. (a) DPV of SWCNT/AuNP modified screen printed gold electrodes modified with the Fc-pepstatin conjugate in (a) the assay buffer, and in the presence of serum samples of HIV-1 infected blood with different replication stages of the virus; at (b) 0; (c) 6.80×10^2; (d) 2.27×10^4; (e) 8.81×10^5; and (f) 1.11×10^6 copies/mL. (b) Correlation between viral replication and the difference of the peak potential shift. Ag/AgCl was used as the reference electrode at 100 mV/s. The assay buffer consisted of 0.1 M sodium acetate, 2 M $NaClO_4$, 1 mM EDTA, 1 mM DTT, and 10% DMSO, pH 7.4. The E° of the Fc/Fc^+ couple under the experimental conditions is 448 (±5) mV.

CONCLUSION

Based on an ultrasensetive electrochemical recognition method of HIV-1 protease, an essential enzyme for virion assembly and maturation, the presence of HIV in serum samples was detected. An inexpensive and disposable screen printed gold electrode was modified with SWCNT/AuNP to increase the exposed surface area of the electrode and therefore enhance its efficiency. The Fc-pepstatin, immobilized on the modified electrode surface as a recognition element, bound specifically to the protease enzyme which was abundantly present in the HIV-1 virus. This proof of concept opens up the search for better-engineered portable microsystems for the early detection of HIV infection that are efficient and sensitive and, more importantly, affordable for developing regions.

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