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Semi-quantification and Potency Verification of the HIV Protease Inhibitor Based on the Matrix-Capsid Protein Immobilized Nickel (II)/NTA-Tol/Graphene Oxide/SPCE Electrochemical Biosensor

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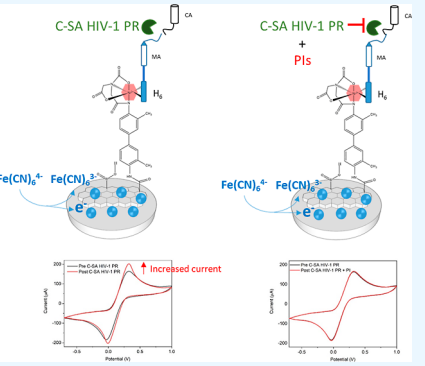
ABSTRACT: Human immunodeficiency virus (HIV) causing acquired immune deficiency syndrome (AIDS) is still a global issue. Long-term drug treatment and nonadherence to medication increase the spread of drug-resistant HIV strains. Therefore, the identification of new lead compounds is being investigated and is highly desirable. Nevertheless, a process generally necessitates a significant budget and human resources. In this study, a simple biosensor platform for semi-quantification and verification of the potency of HIV protease inhibitors (PIs) based on electrochemically detecting the cleavage activity of the HIV-1 subtype C-PR (C-SA HIV-1 PR) was proposed. An electrochemical biosensor was fabricated by immobilizing His6-matrix-capsid (H_6MA-CA) on the electrode surface via the chelation to Ni^{2+}-nitrilotriacetic acid (NTA) functionalized GO. The functional groups and the characteristics of modified screen-printed carbon electrodes (SPCE) were characterized by Fourier transform infrared (FTIR) spectroscopy, scanning electron microscopy (SEM), and energy-dispersive X-ray spectroscopy (EDS). C-SA HIV-1 PR activity and the effect of PIs were validated by recording changes in electrical current signals of the ferri/ferrocyanide redox probe. The detection of PIs, i.e., lopinavir (LPV) and indinavir (IDV), toward the HIV protease was confirmed by the decrease in the current signals in a dose-dependent manner. In addition, our developed biosensor demonstrates the ability to distinguish the potency of two PIs to inhibit C-SA HIV-1 PR activities. We anticipated that this low-cost electrochemical biosensor would increase the efficiency of the lead compound screening process and accelerate the discovery and development of new HIV drugs.



Acquired immunodeficiency syndrome (AIDS) is a disease caused by human immunodeficiency virus1 (HIV-1) infection. Three viral enzymes participate in the HIV-1 replication cycle: reverse transcriptase (RT), protease (PR), and integrase (IN). The proteolytic activity of HIV-1 PR on the precise cleavage of Gag and Gag/polymerase (Pol) precursors generates HIV-1 infectious particles.[1] Accordingly, HIV-1 PR is an essential target for designing the lead compounds to inhibit viral maturation. In highly active antiretroviral therapy (HAART), PIs are one of four antiretroviral drugs recommended in combination for treating HIV-1-infected patients.[2] HIV drug resistance caused by drug adherence failure is still a significant concern. Nowadays, the search for new compounds with effective AIDS therapy, especially for the multidrug resistance strain, is a considerable demand. Since the new HIV-PR mutants have been continuously reported, the discovery of a lead compound consumes a significant amount of budget and labor. Consequently, it takes years to identify a lead compound with a clinical trial potential.[3] Therefore, simplifying screening methods with the ability to compare the inhibitory activity is crucially required.

Mass spectrometry and chromatographic methods, i.e., HPLC-UV, HPLC-MS, and MALDI-MS, are gold standard techniques for the identification and quantification of PIs such as lopinavir (LPV), ritonavir (RTV), and nelfinavir (NFV) in biological fluid samples.[4−7] Although these methods provide several advantages, including high accuracy, great sensitivity and specificity, and simultaneous analysis, their drawbacks are time-consuming analysis, expensive instruments, and the requirement of specialists for operation. In addition, these assays do not support the analysis of the inhibitory activity against HIV-PR. Mammalian cell-based and fluorometric

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assays have been used to monitor the HIV-1 PR activity.[8,9] Since biosafety level 2/3 is obligated for cell-based assays, it is not practical for general laboratories. Previously, we invented an enzyme-linked immunosorbent assay (ELISA)[10−12] and an immunochromatographic (IC) assay for the detection of HIV-1 PR.[13−15] The key success of these assays relies on using a monoclonal antibody (anti-MA mAb, HB8975) to monitor the cleaved form of the matrix-capsid. Although the inherent advantage of ELISA is the sensitivity of detection, several steps and time consumption are the drawbacks. The IC strip test is rapid and more accessible; however, the potency of candidate compounds cannot be evaluated. The sources of lead compounds can be from synthetic chemical libraries[16] or natural products.[17] The assay sensitivity is concerned with the low amount of HIV-PR bioactive compounds from the crude extract of natural products. The biosensor is an interesting platform since the procedure is simple with modest sensitivity. Recently, Miczi et al. developed bio-layer interferometry (BLI) to measure the HIV-1 PR activity.[18] However, BLI is not cost-effective for high-throughput screening and requires a sophisticated instrument.

Certain electrochemical platforms have recently been reported in clinical diagnosis and monitoring. They combine the biorecognition element and transducer for converting the biological event resulting from the reaction between the specific bioreceptor molecule and the analyte into the measurable electrical signal.[19−22] High sensitivity, ease of fabrication, rapid, and low analysis cost conform to the advantages of electrochemical biosensors. Carbon-based and metal nanoparticles were used to improve the sensitivity of the electrochemical sensor. Cyclic voltammetry and electrochemical impedance spectroscopy based on ferrocene (Fc)-pepstatin and single-walled carbon nanotubes/gold nanoparticles (SWCNT/AuNP) modified gold electrode surface were formerly developed for screening the potent PI in binding to the immobilized HIV-1 PR.[23] Another application of this sensor surface was to verify the presence of HIV-1 PR, which reacts with the immobilized peptide.[24] In addition, a reusable electrochemical sensor was developed to quantify trace levels of IDV in actual samples. This sensor used zinc oxide nanorods/molybdenum disulfide nanosheet modified on the screen-printed carbon electrode that exhibited high sensitivity and repeatability toward the sensing of IDV.[25] However, these assays cannot demonstrate the efficiency of compounds in inhibiting the enzymatic activity of HIV-1 PR in the cleavage of the MA-CA substrate.

In this research, we aim to design and fabricate an electrochemical biosensor for PI detection utilizing His6 tagged protein-Ni^{2+}-NTA immobilization. The H_6MA-CA substrate was immobilized via Ni^{2+} coordinated with the synthesized NTA-Tol ligand linked on the GO/SPCE surface. The biosensor fabrication steps were carefully optimized and characterized. The presence of C-SA HIV-1 PR in the test solution resulted in the cleavage of MA-CA residues on the electrode surface, leading to an increment of the electrical current signal of the redox probe. On the other hand, the presence of PIs decreased the signal due to their inhibitory activity against the HIV protease. The developed biosensor demonstrates the property of differentiating the potency of two PIs, i.e., IDV and LPV, against the subtype C-PR. Moreover, this biosensor is a fast, accurate, and inexpensive diagnostic tool in clinical analysis.

EXPERIMENTAL SECTION

Chemicals and Apparatus. All chemical reagents were of analytical reagent grade and were used as received. Deionized (DI) water was obtained from a system of Milli-Q (Millipore, Sweden). To prepare phosphate buffer saline (PBS), 0.24 g of potassium dihydrogen phosphate (KH_2PO_4) (Fisher Scientific, USA), 3.63 g of disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4{\cdot}12H_2O$), 0.2 g of potassium chloride (KCl) (Ajax Finechem, Australia), and 8 g of sodium chloride (NaCl) (Loba Chemie, India) were dissolved in 800 mL DI water. Sodium hydroxide (NaOH) was used to adjust the pH of PBS to 7.4, and DI water was added to 1000 mL. 10 mM $[Fe(CN)_6]^{3-/4-}$ solution was prepared by mixing 10 mM potassium ferrocyanide trihydrate ($K_4[Fe(CN)_6]{\cdot}3H_2O$) and 10 mM potassium ferricyanide ($K_3[Fe(CN)_6]$) (Sigma-Aldrich, Germany) in a ratio of 1:1. Graphene oxide (GO) was prepared by the slightly modified Hummer's method.[26] *N*-(3-dimethylaminopropyl)-*N*′-ethyl carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich (Germany).

The NTA-Tol ligand was synthesized in one pot from nitrilotriacetic acid. To a suspension of nitrilotriacetic acid (1 mmol) in pyridine (0.4 M), acetic anhydride (1.1 mmol) was added. The mixture was heated to 110 °C with stirring for 1 h under an N_2 atmosphere. After cooling to 50 °C, o-tolidine (1.1 mmol) was added and stirred at 110 °C for 1 h. After the completion of the reaction, as justified by thin-layer chromatography (50% EtOAc/hex), the volatiles were removed by a rotary evaporator. Then, water was added to the residue to obtain the precipitate, collected by filtration, and washed several times with water. The crude product was further recrystallized using a mixture of ethanol and water.

A solution of 1 mM of the Ni ion solution was prepared by dissolving 0.0119 g of nickel(II) chloride hexahydrate ($NiCl_2{\cdot}6H_2O$) with 50 mL of DI water. Bovine serum albumin (BSA) was purchased from Sigma-Aldrich. The truncated HIV-1 Gag precursor containing the HIV-1 PR cleavage site (H_6MA-CA) was produced as previously described by Kitidee et al.[12] The purified C-SA HIV-1 PR was kindly provided by Ms. Mpho Setshedi and Prof. Yasien Sayed (University of the Witwatersrand, South Africa).

Three electrodes of SPCEs, consisting of a carbon working electrode (WE) (3.00 mm diameter), auxiliary electrode (AE), and silver/silver chloride (Ag/AgCl) reference electrode (RE), were fabricated in the laboratory as previously reported.[27]

All electrochemical experiments were performed using a PlamSen4 potentiostat (PalmSens BV, Netherlands). The surface of electrodes and their semi-quantitative elemental analysis were performed by a scanning electron microscope (JSM 6335F, JEOL, Japan) equipped with an energy-dispersive X-ray microanalyzer. The Fourier transform infrared (FTIR) spectrum was recorded in the range of 4000−400 cm^{-1} on an FTIR spectrometer (Bruker—Tensor 27) using the attenuated total reflectance (ATR) mode.

HIV-1 PR Subtype C Activity Assay. The production and activity of C-SA HIV-1 PR have been reported previously.[28,29] In brief, C-SA HIV-1 PR was prepared from inclusion bodies with a high yield and specific proteolytic activity after refolding. In this study, the activity of C-SA HIV-1 PR was determined by indirect ELISA. The microtiter plates (Greiner Bio-One, Kremsmünster, Austria) were coated with recombinant H_6MA-CA. Then, C-SA HIV-1 PR was mixed, the reaction solutions

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ACS Omega 2023, 8, 17932−17940

were added into the coated wells, and the plates were incubated at 37 °C for 1 h. The wells were then washed with washing buffer. Then, the wells were detected by an anti-MA mAb (G53) and an anti-MA mAb (HB-8975). Finally, anti-mouse Igs-HRP (KPL, Gaithersburg, MD) was added, and the plates were incubated for 1 h at room temperature. The wells were then washed, followed by the addition of the TMB substrate. The reaction was stopped by 1 M HCl, and the results were measured using a microplate reader at OD 450 nm.

Preparation of Modified Electrodes. The procedure for the modification of screen-printed carbon electrodes is depicted in Figure 1. SPCEs were treated with ozone for 1

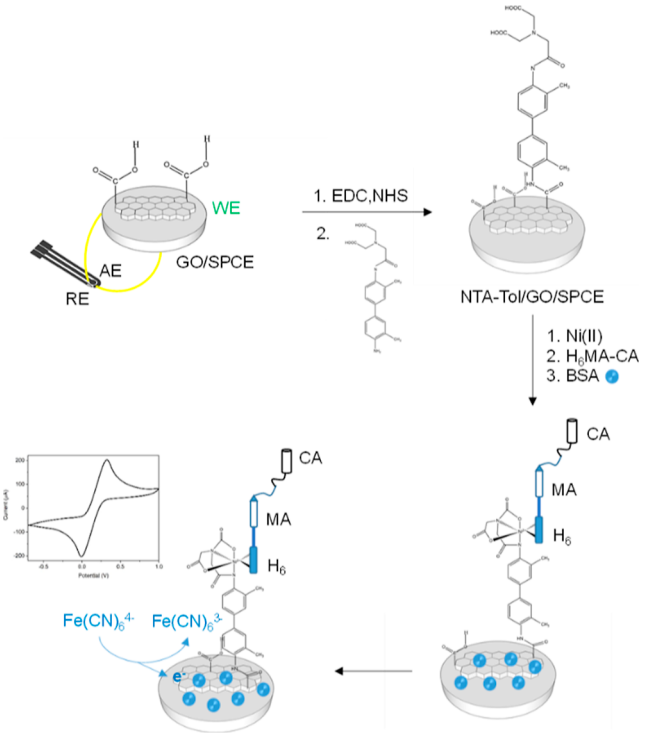

Figure 1. Schematic illustration of the preparation procedure of the modified electrode.

min, except the working area, which was covered with a nail polish coating as an insulator. Then, 20 μL of 1 mg mL^{-1} of the GO suspension was dropped onto the surface of the working electrode (WE) and dried at room temperature, which provided GO/SPCE. Then, a 10 μL mixture of 0.4 M EDC and 0.1 M NHS (1:1) was dropped on electrodes at 4 °C for 30 min to activate the carboxylic acid functional group of GO. Subsequently, the electrode was incubated with 20 μL of the NTA-Tol ligand (1 mM) at 4 °C for 2 h that provided NTA-Tol/GO/SPCE. The Ni^{2+}/NTA-Tol was prepared by dropping 20 μL of the Ni ion solution (1 mM) onto the electrode surface that gave Ni^{2+}/NTA-Tol/GO/SPCE. Next, 30 μL of 1 μg mL^{-1} H_6MA-CA substrate was incubated on Ni^{2+}/NTA-Tol/GO/SPCE at 4 °C for 3 h. After washing with DI water to remove the non-binding of H_6MA-CA, 30 μL of 1 μg mL^{-1} BSA solution was added on the electrode surface to block nonspecific binding sites at 4 °C for 6 h that finally provided BSA/H_6MA-CA/Ni^{2+}/NTA-Tol/GO/SPCE. This modified electrode was kept at 4 °C for later use. The modification of the electrode in each step was confirmed through electrochemical measurements and SEM and EDS characterizations.

To test the activity of C-SA HIV-1 PR, the test solution containing the enzyme was dropped on the electrode surface and incubated at 37 °C for 30 min to cleave the H_6MA-CA substrate and then washed with PBS.

Verification of PIs by the Biosensor. LPV and IDV were selected as representatives of PIs. LPV and IDV were diluted with PBS to the desired concentration. These inhibitors were incubated with C-SA HIV-1 PR in a microtube at room temperature for 1 h. After that, the mixture was dropped on the electrode surface under the same conditions as C-SA HIV-1 PR without an inhibitor to compare the results.

RESULTS AND DISCUSSION

Detection of the Subtype C-PR Activity. C-SA HIV-1 PR was applied for activity assay using an indirect ELISA. C-SA HIV-1 PR was added to the wells coated with recombinant H_6MA-CA. The proteolytic cleavage was carried out under 37 °C for 1 h. The proteolytic activity of C-SA HIV-1 PR was detected using specific antibodies (anti-MA mAb G53 and anti-MA mAb HB-8975). The high OD signal was developed using anti-MA mAb G53 either with or without adding C-SA HIV-1 PR (Figure 2). In contrast, a high OD signal was

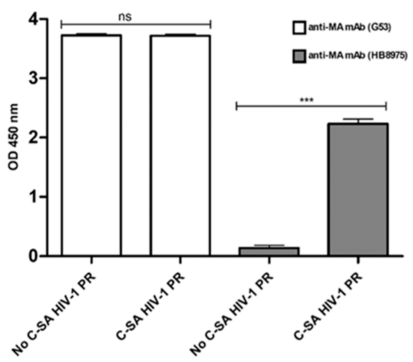

Figure 2. Detection of C-SA HIV-1 PR activity using ELISA. The proteolytic activity of C-SA HIV-1 PR using anti-MA mAb G53 and anti-MA mAb HB-8975 was performed. The bound antibodies were detected using an anti-mouse Igs HRP. After adding the substrate and HCl, the signal was measured at 450 nm. The experiment was performed in triplicates. Statistical significance was determined by a two-tailed Student's test (***$p < 0.0001$).

particularly observed when anti-MA HB-8975 detecting mAb was used in the presence of C-SA HIV-1 PR. This result confirmed the proteolytic cleavage of H_6MA-CA caused by the C-SA HIV-1 PR activity. Thus, the quality of C-SA HIV-1 PR is suitable for further biosensor development.

Principle of the Electrochemical Biosensor for the Screening of PIs. The concept of the electrochemical biosensor for the C-SA HIV-1 PR inhibition assay is presented in Figure 3. The reactions of H_6MA-CA, C-SA HIV-1 PR, and PI that occur on the electrode surface are verified using the cyclic voltammetry (CV) measurement of a ferri/ferrocyanide redox probe. The ferri/ferrocyanide redox probe is useful, resulting in a well-defined oxidative current. As shown in Figure 3A, when the modified electrode is incubated with C-SA HIV-1 PR, the immobilized H_6MA-CA substrate is cleaved and no longer hinders the diffusion of the redox probe to the active area of the electrode. As a result, the current becomes increasingly high. However, the presence of an active PI leads

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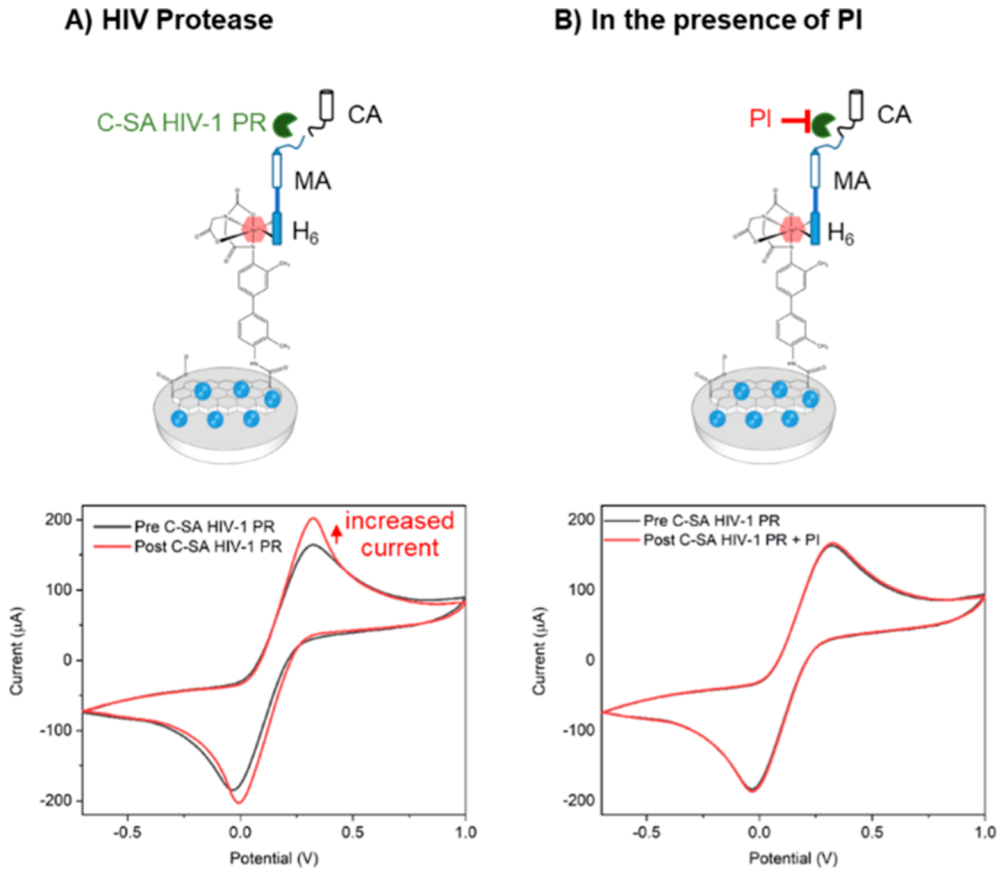

Figure 3. Schematic diagram demonstrating the principle of the electrochemical biosensor for screening PI. (A) In the absence of PI, H_6MA-CA is cleaved by C-SA HIV-1 PR; then the CA is released from H_6MA, resulting in a high measurable current. (B) In the presence of an active PI, the activity of C-SA HIV-1 PR is reduced, thus preventing the cleavage of H_6MA-CA and resulting in low measurable current.

to the reduction of the HIV-PR activity in the processing of the immobilized H_6MA-CA substrate (Figure 3B), and the detected current signal remains intact.

Electrode Characterization. FTIR spectroscopy was used to identify the functional groups of modified electrodes. FTIR spectra of SPCE, GO/SPCE, and NTA-Tol/GO/SPCE are shown in Figure 4. The FTIR spectrum of GO/SPCE consists of absorption peaks at 3431, 1720, 1611, 1375, 1238, and 1074 cm^{-1} corresponding to the O−H stretching vibration, C═O stretching vibration of the carboxyl group, C═O and O−H bending vibration, and C−OH and C−O stretching vibration, respectively. When the GO/SPCE electrode was modified with NTA-Tol via EDC-NHS coupling, the new absorption peak of C−N stretching vibration appeared at 1635 cm^{-1}, indicating that the NTA-Tol was successfully immobilized on GO/SPCE.

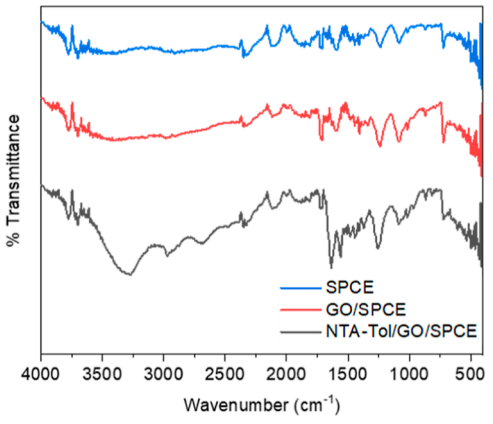

Figure 4. FTIR spectra of SPCE, GO/SPCE, and Tol-NTA/GO/SPCE.

Interrogation of the electrodes in each modification step was performed by scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS). It was found that a bare SPCE has a uniformly rough surface (Figure 5A). After deposition with GO, its surface presents sheets of GO, as shown in Figure 5B. Next, the GO was further functionalized with NTA-Tol via EDC-NHS coupling. The EDS spectrum of NTA-Tol/GO/SPCE and Ni^{2+}/NTA-Tol/GO/SPCE (Figure 5I,J) showed N peak and Ni peak (shown in the circle), respectively. When the substrate H_6MA-CA and BSA blocking agent were immobilized onto the modified electrodes, the electrode surface thickness was increased (shown with the arrows) due to the linked substrate and absorbed blocking agent bound to the electrode (Figure 5E,F).

The aim of this study is to design and fabricate an electrochemical biosensor for PI detection by using His_6-tagged protein-Ni^{2+}-NTA immobilization. The H6MA-CA substrate was immobilized on the GO/SPCE surface via Ni^{2+} coordination with the NTA-Tol ligand. CV has been chosen as the primary technique to demonstrate successful immobilization because it is simple and provides electrochemical characteristics. The cyclic voltammograms of the electrode at different immobilization steps were examined to study the features of each modification step, as shown in Figure 6. When

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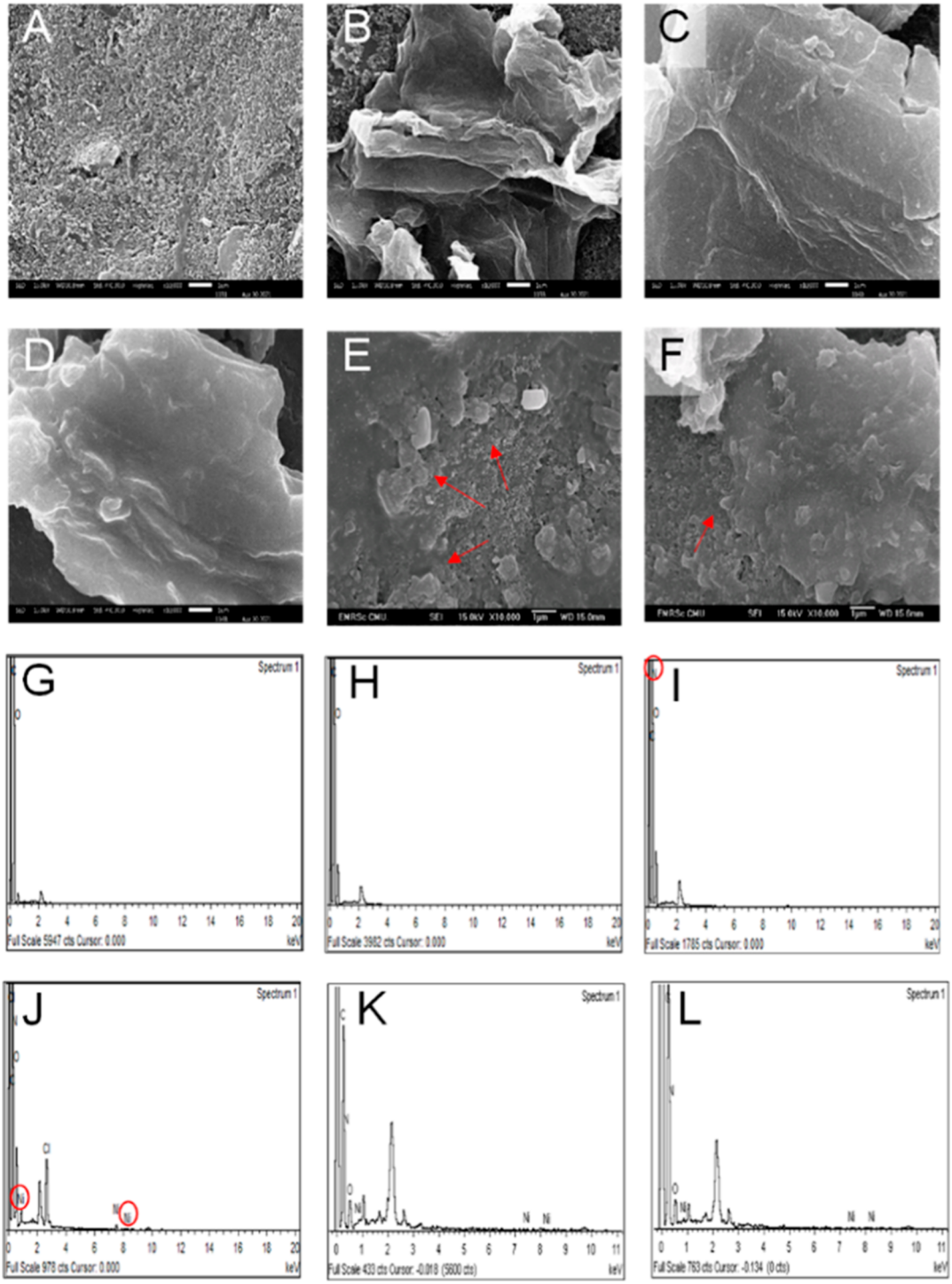

Figure 5. SEM images of (A) SPCE, (B) GO/SPCE, (C) Tol-NTA/GO/SPCE, (D) Ni^{2+}/Tol-NTA/GO/SPCE, (E) MA-CA/Ni^{2+}/Tol-NTA/GO/SPCE, and (F) BSA/MA-CA/Ni^{2+}/Tol-NTA/GO/SPCE. EDS spectrum of (G) SPCE, (H) GO/SPCE, (I) Tol-NTA/GO/SPCE, (J) Ni^{2+}/Tol-NTA/GO/SPCE, (K) MA-CA/Ni^{2+}/Tol-NTA/GO/SPCE, and (L) BSA/MA-CA/Ni^{2+}/Tol-NTA//GO/SPCE.

H_6MA-CA (red line) and BSA (blue line) were immobilized, a decrease in the peak current was observed compared with the CV of the electrode consisting of the Ni^{2+}/NTA functionalized GO/SPCE (black line). It should be noted that the cathodic peak at −0.12 V corresponds to the reduction of ferricyanide, which was found to be quasi-reversible to the oxidation peak observed at 0.55 V. The second cathodic peak at 0.3 V is the reduction of Ni^{2+}, as it appeared only in the presence of Ni^{2+}. The reduction in a peak current is due to the immobilized H6MA-CA and BSA that hinder the electron transfer of the redox probe. In the presence of C-SA HIV-1 PR, the peak current is increased (green line). C-SA HIV-1 PR cleaved H_6MA-CA on the electrode surface to provide MA linked to an electrode surface. The cleaved polypeptides allow more electron transfer between the redox probe and the electrode surface. In selecting nanomaterials to improve analytical sensitivity, another critical consideration for fabricating electrochemical biosensors is the immobilization of the

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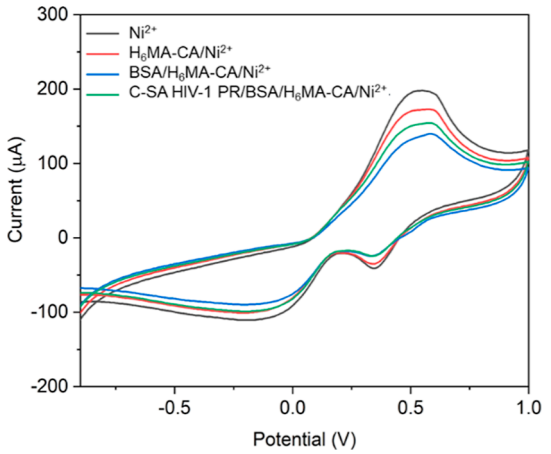

Figure 6. CVs of the different modified electrodes in a 10 mmol L^{-1} ferri/ferrocyanide redox probe at a scan rate of 100 mV s^{-1}.

substrate protein on the working electrode. The immobilization of substrate proteins can be performed via physical adsorption, covalent anchoring or cross-linking, self-assembly monolayer (SAM), and nickel(II)-chelating ligand-His_6 tagged protein. Ni-NTA protocols are often used to purify recombinant proteins containing a polyhistidine sequence.[30] Some reports utilized the nickel(II)-chelating ligand-His_6 tagged protein to immobilize the protein of both the fabrication of biosensor and the ELISA method.[12,31,32] Our result suggested that the H_6MA-CA sensor was successfully fabricated, and this sensor can be applied further for the proteolytic activity of HIV-PR and inhibition of PIs to HIV-PR.

Optimization of the Electrochemical Biosensor. Some parameters of the electrochemical biosensor fabrication were optimized to achieve high detection sensitivity. First, the effect of the amount of the NTA-Tol ligand was studied (Figure 7A). The oxidative current decreased when the amount of NTA-Tol increased from 0 to 10.5 μg mm^{-2}. The decreased current was probably due to the large structure of the NTA-Tol ligand that obstructs the electron transfer of the redox probe. Thus, the optimal amount of NTA-Tol of 10.5 μg mm^{-2} was chosen. Next, the amount of Ni^{2+} on the electrode surface was studied (Figure 7B). The oxidation peak current was increased when increasing the amount of Ni^{2+} because of the benefits of electrostatic interaction between the positive charge of Ni^{2+} and the negative charge of the ferri/ferrocyanide redox probe. The oxidative current of the redox probe increased until the amount of Ni^{2+} reached 0.67 μg mm^{-2}. The Ni^{2+} amount of 0.67 μg mm^{-2} was selected for modifying the electrode surface. The optimal amounts of the H_6MA-CA substrate and the BSA blocking agent were investigated. It was found that the oxidation peak current decreased with increasing amount of the H_6MA-CA substrate and the BSA blocking agent. We reasoned that the large biomolecule of H_6MA-CA and BSA impeded the electron transfer of the redox probe. Consequently, an amount of 4.2 ng mm^{-2} for the H_6MA-CA substrate and the BSA blocking agent was selected for immobilization on the electrode surface.

Having an electrochemical biosensor established, we next investigate the ability of this biosensor to detect HIV protease

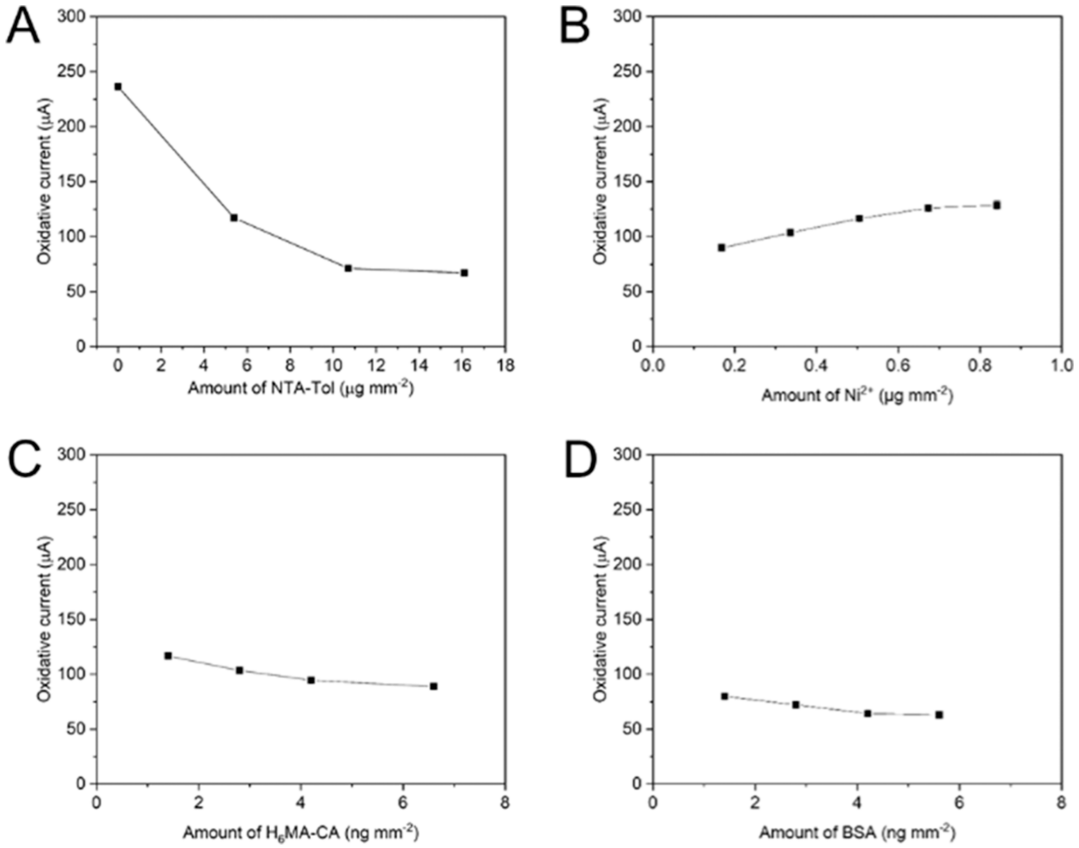

Figure 7. Relationship between the oxidative peak current and different amounts of (A) NTA-Tol ligand, (B) Ni^{2+}, (C) H_6MA-CA substrate, and (D) BSA blocking agent.

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(C-SA HIV-1 PR) activities. C-SA HIV-1 PR concentrations of 10 and 100 μg mL^{-1} were chosen in our studies. To monitor the cleavage of the H_6MA-CA substrate, the electrodes were measured for cyclic voltammetry both before and after treatment with C-SA HIV-1 PR. The current response before and after the C-SA HIV-1 PR incubating step was expressed as the percentage of increasing current where I_0 and I_p denote the peak current response before and after C-SA HIV-1 PR incubation steps, respectively.

$$\text{Increasing current (\%)} = \frac{Ip - I_0}{I_0} \times 100$$

The increasing current percentage is higher in the presence of C-SA HIV-1 PR. The increasing current percentage was found to be 5.5 ± 0.7% at 10 μg mL^{-1} C-SA HIV-1 PR. The current percentage increased to 12.7 ± 1.4% at 100 μg mL^{-1} C-SA HIV-1 PR incubation. The C-SA HIV-1 PR concentration of 100 μg mL^{-1} was selected for further experiment.

Confirmation of H_6MA-CA Immobilization via Immunoreaction. Immunoreaction is necessary to investigate the presence of H_6MA-CA on the electrode surface. Therefore, anti-MA mAb (G53) was chosen as a specific antibody, and anti-interferon-γ (anti-IFN-γ mAb) was used as an irrelevant antibody. The result is shown in Figure 8. The oxidative peak

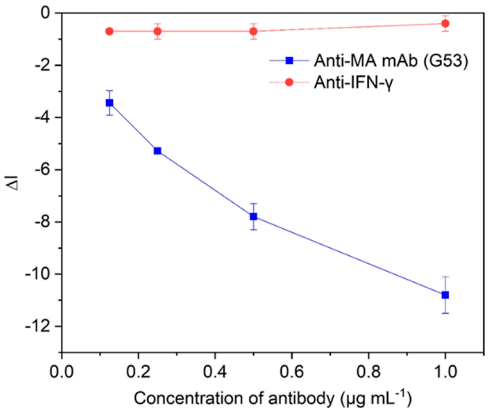

Figure 8. Relationship between the oxidative peak current and concentration of the antibody. ΔI was calculated from oxidative current antibody/BSA/Ni^{2+}/NTA-Tol/EDC-NHS/GO/SPCE−oxidative current BSA/Ni^{2+}NTA-Tol/EDC-NHS/GO/SPCE.

current decreased when the anti-MA mAb (G53) concentration increased. In contrast, when the electrode was incubated with anti-IFN-γ mAb, the oxidative peak current was comparable to that without anti-IFN-γ mAb. Accordingly, the substrate H_6MA-CA was successfully occupied on the modified electrode.

Screening for the HIV-PR Inhibitor/Drug Assay. The practical applicability of the electrochemical biosensor has been confirmed by investigating the inhibition of HIV-PR by LPV and IDV inhibitors. The developed biosensor was incubated with C-SA HIV-1 PR compared to a mixture of C-SA HIV-1 PR and PIs. The percentage of the increasing current of each electrode is shown in Figure 9. In the presence of PIs, the percentage of the increasing current decreased in all cases and in a dose-dependent manner. LPV and IDV inhibited C-SA HIV-1 PR, which prevented the H_6MA- CA substrate on the electrode surface from cleavage. The result showed that IDV at 1 and 10 μg mL^{-1} could abolish the proteolytic activity

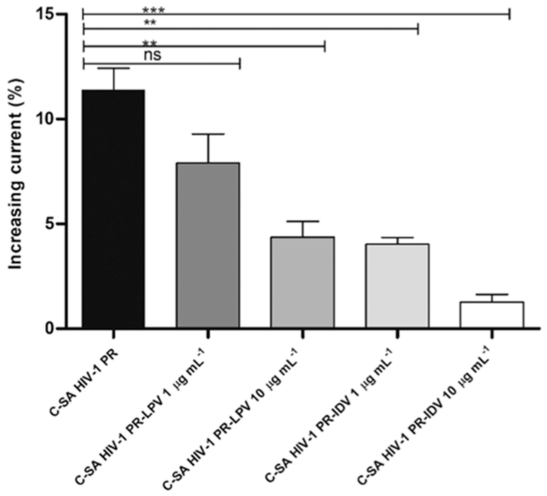

Figure 9. Percentage of increasing current of the absence and presence of PI. The experiment was performed in triplicate. Statistical significance was determined by a two-tailed Student's test (***p < 0.0001).

of C-SA HIV-1 PR higher than LPV. The susceptibility to PI has been investigated in various HIV-1 subtypes. The study reported that the HIV subtype C was highly susceptible to IDV.[33] This evidence complies with our findings, which deliberate the susceptibility of C-SA HIV-1 PR to LPV less than IDV. These results confirm that the proposed biosensor could be a practical approach to screening PIs.

CONCLUSIONS

We have developed an in vitro platform for measuring the HIV-PR activity by immobilizing modified SPCE with an H_6MA-CA substrate. The H_6MA-CA substrate was anchored on the SPCE surface via Ni^{2+} coordinating with the NTA-Tol functionalized GO. This electrochemical biosensor can discriminate the presence of PIs at different levels by detecting the percentage of current changes. At higher LPV and IDV concentrations, the percentage of current changes was smaller. Apart from the semi-quantification of PI levels, the ability to inhibit the HIV-PR activity was interpreted in a single step. Accordingly, it will be a platform for searching for novel PIs to impede the propagation of HIV-PR mutants resisting current therapeutic drugs. Therefore, identifying PIs from synthetic chemical libraries or crude extracts from natural sources is feasible in a shorter time. Our strategy can be applied to the discovery of inhibitors for other HIV enzymes, i.e., integrase and reverse transcriptase. Furthermore, the immobilization technique is superior to other methods, i.e., physical adsorption or covalent coupling, since the bonding interaction is more specific. In addition, the immobilization process can be proceeded under mild conditions, thus preserving the quality of the anchored protein. This platform of immobilizing substrate H_6MA-CA via Ni-NTA on the electrode surface provides a prototype for immobilizing other target proteins containing six histidine residues.

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The manuscript was written through contributions of all authors. W.T. and P.K. contributed equally. W.T. and P.K. performed the experiments, validated and analyzed the experiment results, and wrote the manuscript. C.T. conceived the research idea and reviewed and edited the manuscript. W.K., J.J., N.S., M.S., and Y.S performed the experiments and reviewed and edited the manuscript. C.T., W.K., and J.J. obtained the funding and designed the experiment. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; PIs, HIV protease inhibitors; C-SA HIV-1 PR, HIV-1 subtype C-PR; H_6MA-CA, His6-matrix-capsid; SPCE, modified screen-printed carbon electrodes; FTIR, Fourier transform infrared; EDS, energy-dispersive X-ray spectroscopy; LPV, lopinavir; IDV, indinavir; NFV, nelfinavir; RT, reverse transcriptase; PR, protease; IN, integrase;; HAART, highly active antiretroviral therapy; ELISA, enzyme-linked immunosorbent assay; IC assay, immunochromatographic (IC) assay; mAb, monoclonal antibody; BLI, bio-layer interferometry; SWCNT/AuNP, single-walled carbon nanotubes/gold nanoparticles; SAM, self-assembly monolayer.

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