An electrochemical approach for the detection of HIV-1 protease†

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Received (in Cambridge, UK) 11th May 2007, Accepted 29th June 2007
First published as an Advance Article on the web 13th July 2007
DOI: 10.1039/b707140j

An electrochemical biosensor with a new bioorganometallic approach for the detection of human immunodeficiency virus (HIV) type 1 protease (HIV-1 PR) using surface-bound ferrocenoyl (Fc)–pepstatin conjugates is presented.

It has been more than 20 years since HIV was first identified as the causative agent of AIDS.[1] One of the current pathways to develop inhibitors that target different steps in the life cycle of the virus is blocking the function of the HIV-1 PR, which is an essential enzyme required in the proper assembly and maturation of infectious virions. HIV-1 PR cleaves the viral polyprotein precursors into individual mature proteins. If the activity of HIV-1 PR is inhibited, the budded immature viral particles that contain inactive HIV-1 PR cannot undergo maturation to an infective form.[2] Thus, the necessity of this enzyme in the virus life cycle has put it into the limelight as a promising target for the therapy of AIDS. A global effort and intensive research have been focused on the development of effective HIV inhibitors.[2]

HIV-1 PR is a 99-amino acid aspartyl protease, which functions as a homodimer with only one active site.[3] Characteristically, the enzymes of this family are inhibited by pepstatin, which contains two residues of the unusual amino acid statine.[2–4] Pepstatin was shown to bind to the active sites of most aspartic proteases with unusually small dissociation constants (4.57×10^{-11} M in the case of pepsin).[2] The X-ray analysis of HIV-1 PR confirmed the structural homology among retroviral enzymes.[3]

Optical biosensor technology based on surface plasmon resonance (SPR) has been applied in the screening of compounds interacting with HIV-1 PR.[5] In the SPR approach, HIV-1 PR was immobilized on the sensor surface and the binding of a variety of inhibitors with HIV-1 PR was monitored in a continuous flow. Bioluminescence resonance energy transfer has recently been employed for the detection of HIV-1 PR inhibitors.[6]

In this report, we are taking an electrochemical approach to the detection of HIV-1 PR, making use of a ferrocene (Fc)-conjugated pepstatin,[7] bioconjugate-3 as shown in Fig. 1, that can be readily attached to a gold surface. The bioconjugate-3 was immobilized on screen-printed gold electrodes (SPEs) by immersing them in a 1 mM solution of the peptide in ethanol. After overnight incubation (~15 h), the electrodes were rinsed with distilled water. Diluted films were prepared by following the above procedure with an additional step of incubating the modified electrodes in 1 mM ethanolic solution of hexanethiol for 10 min, followed by stringent rinsing with ethanol. Cyclic voltammograms (CVs) were recorded in aqueous solutions of 2 M $NaClO_4$. Integration of the faradaic peak currents of the CV curves allowed us to evaluate the surface concentration of the film covalently linked to the surface by an Au–S bond.

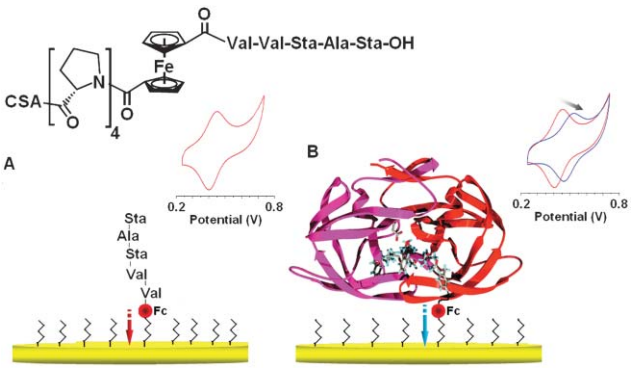

Fig. 1 (A) Chemical drawing of the ferrocenoyl (Fc)-conjugated pepstatin (bioconjugate-3) and the schematic representation of the diluted self-assembled peptide film on a gold surface. The cyclic voltammogram of Fc is observed as shown in red curves. (B) The binding of HIV-1 PR to the bioconjugate-3 causes a significant shift in the formal potential and a decrease in the current intensity as shown in blue curves.

The stock solution of HIV-1 PR (200 nM) was prepared using 0.1 M sodium acetate (pH 4.7) with 2 M $NaClO_4$, 1 mM EDTA, 1 mM dithiothreitol (DTT), 10% dimethyl sulfoxide (DMSO). Fig. 2A shows the changes of the CV responses as HIV-1 PR concentration increased. The peak current from the CVs increased

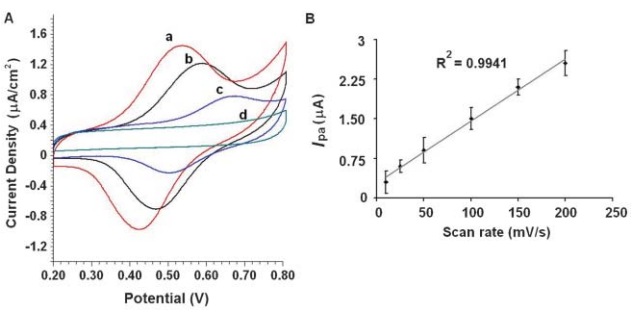

Fig. 2 (A) Cyclic voltammograms of SPEs modified with bioconjugate-3 in the presence of increasing concentrations of HIV-1 PR at (a) 40 nM, (b) 60 nM, (c) 100 nM and (d) 100 nM in the absence of bioconjugate-3 at a scan rate of 100 mV s^{-1}. HIV-1 PR assay buffer included 0.1 M sodium acetate with 2 M $NaClO_4$, 1 mM EDTA, 1 mM DTT, 10% DMSO (pH 4.7). (B) Linear relationship between the scan rate and the anodic peak current for bioconjugate-3 film.

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† Electronic supplementary information (ESI) available: Experimental details of the peptide synthesis, electrode modification and electrochemical measurements. See DOI: 10.1039/b707140j

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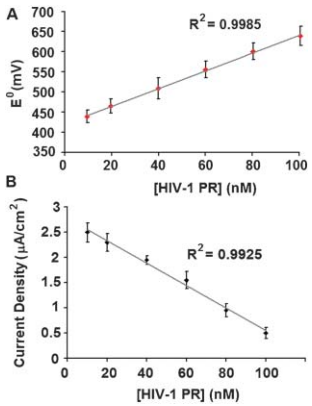

Fig. 3 Plots for the linear relationship between HIV-1 PR concentration and (A) the formal potential (E°); (B) the current density. Other conditions were as described in Fig. 2.

linearly with scan rate (Fig. 2B) as is expected for an adsorbed film.[8]

As the enzyme concentration increased, the formal potential for the surface-bound bioconjugate-3 shifted to higher potentials, indicating that the oxidation of the Fc group was becoming increasingly more difficult, as HIV-1 PR was binding to pepstatin and encapsulating the Fc redox center on the surface. This interaction was plotted with E° as a function of HIV-1 PR concentration (Fig. 3A). A linear relationship was observed for HIV-1 PR concentration up to 100 nM. Afterwards, the potential reached a steady state indicating potentially the saturation of the surface with HIV-1 PR. The detection limit was 1 nM estimated from $3(S_b/m)$, where S_b is the standard deviation of the measurement signal for the blank and m the slope of the analytical curve in the linear region.[9]

A linear decrease in the signal intensity was observed (Fig. 3B). The less efficient penetration of the peptide film by the supporting electrolyte upon binding of the bulky HIV-1 PR on the surface decreased the ability of Fc moieties to be oxidized. Thus, we are suggesting that both the decrease in the signal intensity and the anodic shift in the formal potential can be utilized for analytical evaluation of the biosensor.

Control experiments were performed using pepsin in order to test non-specific binding. Incubation of electrodes modified with bioconjugate-3 with solutions of pepsin was carried out at various pH. As shown in Fig. 4, the binding process was greatly affected by the changes in the pH of the solution.

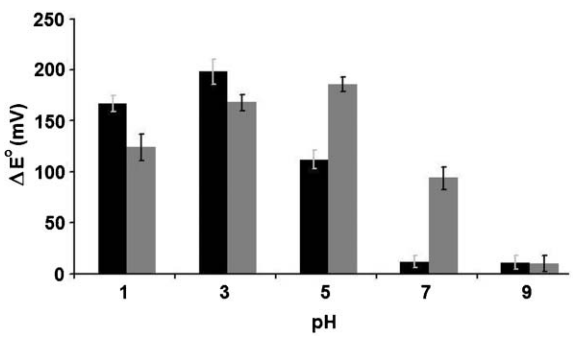

Fig. 4 Column chart for the effect of pH on the interaction between bioconjugate-3 and two target proteins, pepsin (black) and HIV-1 PR (gray). Error bars indicate standard deviation (n = 3).

Interactions with pepsin are observed only in a low pH regime that enables pepsin to potentially bind to the Fc–pepstatin surface. At a pH > 6, pepsin is not stable,[10] and no interactions with the surface-bound bioconjugate-3 were detectable above pH 6. For the interaction with HIV-1 PR, shifts of the E° were observed at pH < 9, with an optimal binding at pH 5. Thus, it is possible to discriminate between bindings of these two proteins to the bioconjugate-3 using electrochemical methods.

Human serum albumin (HSA) was used to test the specificity of our approach. The electrochemical test was performed using 75 μM HSA, and the resulting signals were compared with that obtained with the spiked concentrations of HIV-1 PR, as shown in Fig. 5A. These experiments demonstrated the high specificity of the assay since alterations in the peak potential and current density were obtained only when HIV-1 PR was present, whereas the changes were negligible in the presence of HSA or of the blank solution. Human serum, diluted 10 times, was also tested alone or spiked with HIV-1 PR, and the results were also compared with the same concentrations prepared in buffer. Fig. 5B shows that comparable responses were found for both buffer containing HSA and the serum samples. A weak matrix effect was observed considering the lower currents measured in serum with respect to buffer containing HSA.

We have also tested a well-described inhibitor of HIV-1 PR using our biosensor (Fig. 6A). Cytochalasin A is a cell-permeable fungal toxin that is an oxidized derivative of cytochalasin B. Cytochalasin A inhibits actin polymerization and interferes with microtubule assembly by reacting with sulfhydryl groups.[11] Unlike the other members of the cytochalasins, cytochalasin A acts as an inhibitor of HIV-1 protease.[11] It was reported that the HIV-1 protease activity displayed an IC_{50} of 3 μM and the mode of inhibition was competitive with respect to pepstatin (apparent K_i = 1 μM).[11] As the concentration of cytochalasin A increased, the shift in the peak potential became significantly small (Fig. 6B) in comparison with the one obtained in the absence of the inhibitor, and the current density response increased (Fig. 6C). The agreement between our results and the ones reported for the

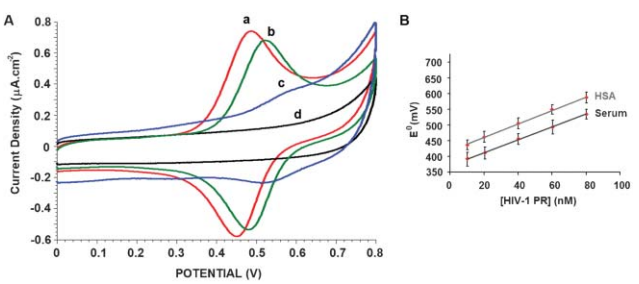

Fig. 5 (A) Cyclic voltammograms of SPEs modified with bioconjugate-3 with increasing concentrations of HIV-1 PR at (a) 20 nM, (b) 40 nM in the presence of 75 μM HSA in the buffer solution, (c) 100 nM HIV-1 PR spiked in 10-times diluted human serum, and (d) only 10-times diluted human serum with no bioconjugate-3 immobilized on the surface at a scan rate of 100 mV s^{-1}. (B) The dependence of the anodic peak potential on the concentration of HIV-1 PR spiked in the assay buffer solution including 75 μM HSA (gray line), and in the 10-times diluted human serum (black line). Other conditions were as described in Fig. 2.

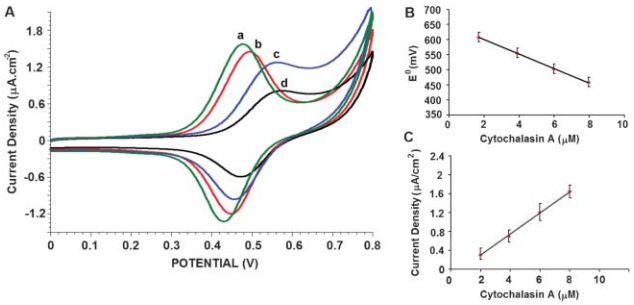

Fig. 6 (A) Cyclic voltammograms of SPEs modified with bioconjugate-3 in the presence of increasing concentrations of the inhibitor, cytochalasin A at (a) 8 μM, (b) 7 μM, (c) 6 μM and (d) 2 μM in the presence of 100 nM HIV-1 PR at a scan rate of 100 mV s^{-1}. Plots for the linear relationship between inhibitor concentration and (B) E° and (C) current density. Other conditions were as described in Fig. 2.

biological activity of cytochalasin A opens a promising platform for the screening of other possible protease inhibitors.

This report demonstrates the proof-of-concept of our electrochemical approach showing that Fc–peptide conjugates bound to a transducer surface hold promise for the electrochemical detection of clinically important proteins that have no redox-active centers, such as telomerase and prostate-specific antigen. We have also investigated the possibility of detecting HIV-1 PR in biological fluids as well as in the presence of other interfering proteins such as HSA. We suggest that this general procedure can be applied in the future to other analytes in complex matrixes. We are currently working on the adaptation of our system into a microarray format[12] that will enable the multiplexed detection of several HIV-related proteins, such as the trans-activator transcription (TAT) protein,[13] as well as the high-throughput screening of candidate inhibitors of HIV-1 PR.[14]

Financial support from NSERC is gratefully acknowledged. H.-B. K. holds the Canada Research Chair in Biomaterials. We are grateful to the Saskatchewan Structure Science Centre (SSSC), to Prof. David A. R. Sanders in the Department of Chemistry and to the Department of Biochemistry for providing access to their instrumentation and reagents.

Notes and references

1 (*a*) F. Barre-Sinoussi, J.-C. Chermann, F. Rey, M. T. Nugeyre, S. Chamaret, J. Gruest, C. Dauguet, C. Axler-Blin, F. Brun-Vezinet, C. Rouzioux, W. Rozenbaum and L. Montagnier, *Science*, 1983, **220**, 868–871; (*b*) R. C. Gallo and L. Montagnier, *Sci. Am.*, 1988, **259**, 40–48; (*c*) R. A. Kramer, M. D. Schaber, A. M. Skalka, K. Ganguly, F. Wong-Staal and E. P. Reddy, *Science*, 1986, **231**, 1580–1584; (*d*) J. R. Huff, *J. Med. Chem.*, 1991, **34**, 2305–2314; (*e*) A. Brik and C.-H. Wong, *Org. Biomol. Chem.*, 2003, **1**, 5–14.

2 (*a*) A. Wallqvist, R. L. Jernigan and D. G. Covell, *Protein Sci.*, 1995, **4**, 1881–1903; R. E. Babine and S. L. Bender, *Chem. Rev.*, 1997, **97**, 1359–1472; (*b*) S. Piana, D. Sebastiani, P. Carloni and M. Parrinello, *J. Am. Chem. Soc.*, 2001, **123**, 8730–8737; (*c*) A. D. Richards, R. Roberts, B. M. Dunn, M. C. Graves and J. Kay, *FEBS Lett.*, 1989, **247**, 113–117; (*d*) L. de Witte, A. Nabatov, M. Pion, D. Fluitsma, M. A. W. P. de Jong, T. de Gruijl, Y. van Kooyk, V. Piguet and T. B. H. Geijtenbeek, *Nat. Med.*, 2007, **13**, 367–371; (*e*) J. Münch, L. Ständker, K. Adermann, A. Schulz, M. Schindler, R. Chinnadurai, S. Pöhlmann, C. Chaipan, T. Biet, T. Peters, B. Meyer, D. Wilhelm, H. Lu, W. Jing, S. Jiang, W.-G. Forssmann and F. Kirchhoff, *Cell*, 2007, **129**, 263–275.

3 H. Toh, M. Ono, K. Saigo and T. Miyata, *Nature*, 1985, **315**, 691.

4 R. Lapatto, T. Blundell, A. Hemmings, J. Overington, A. Wilderspin, S. Wood, J. R. Merson, P. J. Whittle, D. E. Danley, K. F. Geoghegan, S. J. Hawrylik, S. E. Lee, K. G. Scheld and P. M. Hobart, *Nature*, 1989, **342**, 299–302.

5 (*a*) P. O. Markgren, M. Hamalainen and U. H. Danielson, *Anal. Biochem.*, 2000, **279**, 71–78; (*b*) P.-O. Markgren, M. T. Lindgren, K. Gertow, R. Karlsson, M. Hamalainen and U. H. Danielson, *Anal. Biochem.*, 2001, **291**, 207–218; (*c*) M. Alterman, H. Sjobom, P. Safsten, P.-O. Markgren, U. H. Danielson, M. Hamalainen, S. Lofas, J. Hulten, B. Classon, B. Samuelsson and A. Hallberg, *Eur. J. Pharm. Sci.*, 2001, **13**, 203–212; (*d*) C. F. Shuman, P.-O. Markgren, M. Hamalainen and U. H. Danielson, *Antiviral Res.*, 2003, **58**, 235–242; (*e*) H. Kitano, Y. Makino, H. Kawasaki and Y. Sumi, *Anal. Chem.*, 2005, **77**, 1588–1595.

6 K. Hu, J.-F. Clement, L. Abrahamyan, K. Strebel, M. Bouvier, L. Kleiman and A. J. Mouland, *J. Virol. Methods*, 2005, **128**, 93–103.

7 Y. Xu and H.-B. Kraatz, *Tetrahedron Lett.*, 2001, **42**, 2601–2603.

8 H. O. Finklea, in *Electroanalytical Chemistry*, ed. A. J. Bard and I. Rubinstein, Marcel Dekker, New York, 1993, vol. 19, pp. 110–335.

9 J. C. Miller and J. N. Miller, in *Ellis Horwood Series in Analytical Chemistry*, ed. R. A. Chalmers and M. Masson, Chichester, 1993, p. 119.

10 A. Cornish-Bowden and J. Knowles, *Biochem. J.*, 1969, **113**, 353–362.

11 (*a*) A. W. Dobrowski, G. F. Bills, G. Sabnis, L. R. Koupal, R. Meyer, J. G. Ondeyka, R. A. Giacobbe, R. L. Monaghan and R. B. Lingham, *J. Antibiot.*, 1992, **45**, 671–678; (*b*) J. Ondeyka, O. D. Hensens, D. Zink, R. Ball, R. B. Lingham, G. Bills, A. Dobrowski and M. Goetz, *J. Antibiot.*, 1992, **45**, 679–685.

12 K. A. Mahmoud and H.-B. Kraatz, *Chem.–Eur. J.*, 2007, **13**, 5885–5895.

13 (*a*) M. Minunni, S. Tombelli, A. Gullotto, E. Luzi and M. Mascini, *Biosens. Bioelectron.*, 2004, **20**, 1149–1156; (*b*) S. Tombelli, M. Minunni, E. Luzi and M. Mascini, *Bioelectrochemistry*, 2005, **67**, 135–141.

14 V. Hornak and C. Simmerling, *Drug Discovery Today*, 2007, **12**, 132–138.