analytical chemistry

Letter

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Homogeneous Electrochemical Assay for Protein Kinase Activity

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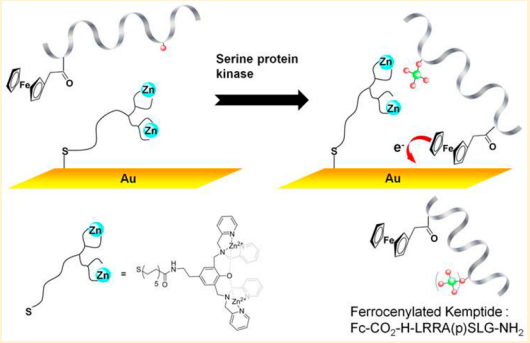
Supporting Information

ABSTRACT: Herein, we report a homogeneous assay for protein kinase activity using an electrochemistry-based probe. The approach involves a peptide substrate conjugated with a redox tag and the phosphate-specific receptor immobilized on an electrode surface. The peptide substrate phosphorylated by a protein kinase binds to the receptor site of the probe, which results in a redox current under voltammetric measurement. Our method was successfully applied even in the presence of citrated human blood and modified to enable a single-use, chip-based electrochemical assay for kinase activity.



The phosphorylation of proteins regulates almost all aspects of cellular function, while abnormal phosphorylation is recognized as a cause or consequence of many diseases.[1−3] About 30% of human proteins are modified by phosphorylation, and approximately 518 protein kinases, the enzymes responsible for controlling protein phosphorylation, are encoded by the human genome.[4] Mutations in particular protein kinases give rise to a number of disorders and many naturally occurring toxins and pathogens exert their effects by altering the phosphorylation states of intracellular proteins.[5−7] Understanding the specificity and regulation of a particular protein kinase is crucial for the development of drugs to treat diseases such as diabetes or cancer.

Tremendous efforts have been devoted to quantification of the catalytic activities of these enzymes. The incorporation of radioactive phosphate from [γ-^{32}P] adenosine 5′-triphosphate (ATP) into protein or peptide substrates is the most common method for measuring cellular kinase activities.[8−10] Recently, a homogeneous kinase activity assay based on fluorescent probes was reported.[11−14] We also previously described a simple and versatile strategy for monitoring kinase activity using phosphate-selective fluorescent quenchers.[15,16] We showed that these synthetic quencher probes could be used to detect Abelson kinase activity and to diagnose chronic myelogenous leukemia in patient samples.

Kinase activity assays based on electrochemical methods have also been developed, enabling rapid, cost-effective, and high-throughput screening of anticancer drugs.[17−19] Herein, we demonstrate a homogeneous assay for protein kinase activity using synthetic receptors immobilized on an electrode surface. While most previous reports were based on a heterogeneous reaction in which the enzyme phosphorylates a peptide substrate fixed on a solid surface, our method employs homogeneous enzymatic reactions where the enzyme reacts with a dissolved substrate in aqueous media. In our assay, the receptor part immobilized on the electrode selectively captures the phosphorylated peptide in solution, producing the electrochemical signal. Briefly, the probe consists of a phosphorylated peptide-binding receptor with a high-affinity binding site at one end and a thiol group at the other that enables self-assembly onto the surface of the gold electrode through its thiol end to generate the electrochemical readout system. Recognition of phosphorylated peptides by receptors immobilized on the surface of the electrode results in an electrochemical signal. A schematic diagram of the kinase assay is presented in Figure 1. The protein kinase phosphorylates the peptide substrate in the enzyme reaction mixture, and then the mixture solution is introduced to the modified electrode surface. The enzymatic product, bound to the surface through the binuclear Zn^{2+} complex, generates an electrochemically oxidative signal from its ferrocenyl conjugate when placed under potential, efficiently reporting the enzymatic catalysis.[20−23] cAMP-dependent protein kinase (PKA), a typical serine/threonine protein kinase, was used as a model enzyme in our study. Ferrocenylated kemptide (Fc-CO-LRRApSLG-NH2), a commercially known PKA-specific peptide, was used both as the phosphorylation substrate and as the reporter for the PKA activity. The voltammetric study confirmed that the higher the concentration of the enzyme, the more the phosphorylated product bound to the receptor part of the probe. One concern with assays of this type is that the phenoxo-bridged bis(Zn^{2+}−dipicolylamine) complex in the

Received: July 11, 2014
Accepted: October 23, 2014
Published: October 23, 2014

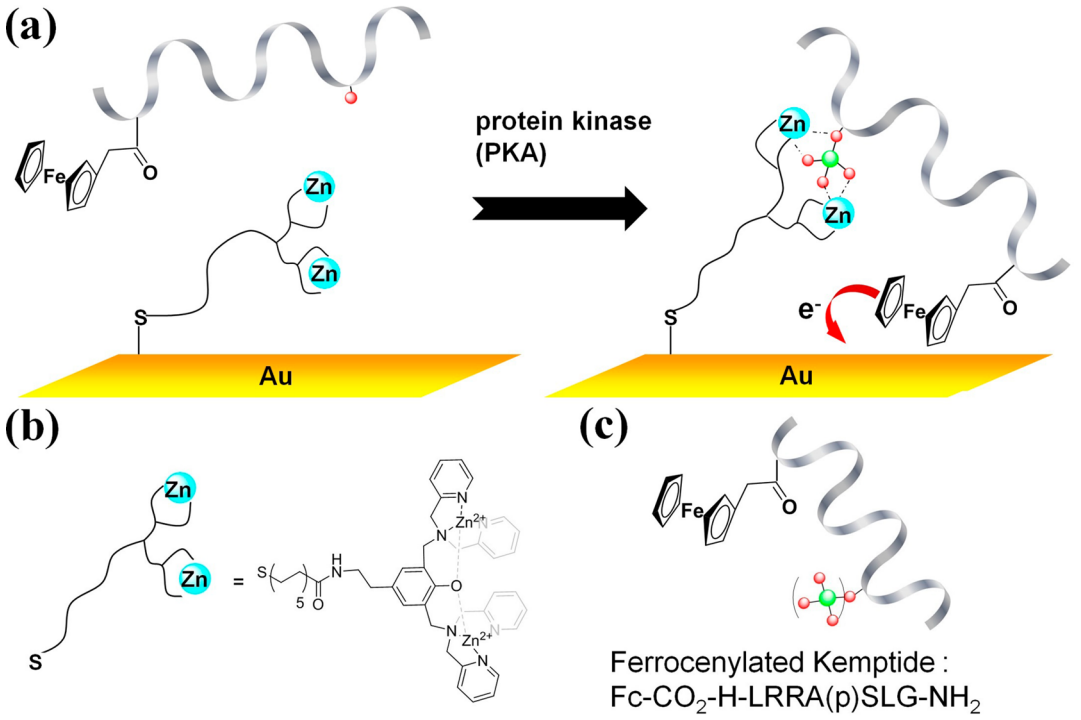


Figure 1. (a) Schematic strategy of homogeneous electrochemical assay for protein kinase activity, (b) structure of the phosphorylated peptide-binding receptor, and (c) ferrocenylated kemptide was used as a peptide substrate for PKA.

probe can bind to ATP and adenosine 5′-diphosphate (ADP), which are the substrate and the byproduct of the enzymatic reaction, respectively. Thus, these binding events can interfere with monitoring the protein kinase activity. However, a large increase in voltammetric current was observed upon phosphorylation of the peptide substrate, which is presumably because the binding affinity of the electrochemical probe to the enzymatic product was sufficiently strong under the tested conditions. Therefore, the homogeneous electrochemical kinase assay was successful as performed in our approach. Previously, a fluorescent probe based on a bis(Zn^{2+}−dipicolylamine) moiety also successfully monitored the enzymatic phosphorylation in buffer solution where there were other potentially interfering phosphate groups such as ATP and ADP.[15,16]

In order to determine the time for probe modification of the electrode surface, a series of cyclic voltammetric (CV) measurements were undertaken in the presence of potassium ferrocyanide ($K_4Fe(CN)_6$) with different lengths of deposition time of the probe solution (Figure S1 in the Supporting Information). These experiments showed that an immobilization time of 12 h was enough and was therefore used to carry out further studies. A mixture of 40 μM peptide substrate, 240 μM ATP, and different concentrations of PKA in the presence of 1× PKA buffer was incubated at 37 °C for 1 h to phosphorylate the substrate. The phosphorylated product without any filtration was then introduced to the probe-modified electrode for 30 min. During this time, the phosphate group of the product was expected to bind to the probe surface generating a current signal from its ferrocenyl branch.

For the enzymatic assay, a differential pulse voltammetric (DPV) study was carried out to measure changes in the electrochemical signal by enzymatic catalysis. The DPV study was performed in a scan range of 0−0.5 V, with an increment of 0.002 V, amplitude of 0.05 V, and pulse width of 0.04 s. In Figure 2a, the mixture produced a well-defined increasing peak current at 0.28 V (vs Ag/AgCl) corresponding to the electrochemical oxidation of the ferrocenyl conjugate in association with the increase in the PKA concentration (0.1−40 U/mL). These current peak signal results suggest that higher measured current corresponds with increased formation of the phosphorylated product in the presence of higher levels of the PKA enzyme. Indeed, several control experiments confirmed that this signal originated from the product of the enzymatic reaction. The peak current appeared and increased only when peptide possessing both the ferrocenyl conjugate and the phosphate group simultaneously existed in the solution. No significant differential current peak was observed from the substrate alone. Figure 2b exhibits the isothermal enzymatic activity curve plotted using the data collected during the DPV measurements of the ferrocenyl conjugate at various PKA concentrations. The detection limit of PKA was estimated to be 0.1 U/mL (S/N = 3, n = 5) with a relatively linear increase from 5 U/mL to 50 U/mL whereas no signal was obtained from the product incubated in the absence of PKA. This result not only signifies that the signal change is not related to nonspecific adsorption of the peptide substrate on the electrode surface but also proves that the kinase indeed phosphorylates the peptide, resulting in a readily detectable increase in the redox current in a highly specific manner.

In order to further demonstrate the potential application of this method, a series of experiments were performed using human blood samples. Human blood was freshly drawn from a donor and citrated to prevent coagulation. Citrated blood was first filtered using a 0.45 μm syringe filter and then combined with the reaction mixture containing different concentrations of PKA and incubated as described above. The enzymatic reaction mixture was then subjected to voltammetric measurements using the probe-modified electrode, and the oxidation current of the ferrocenyl conjugate on the phosphorylated product was measured with DPV. The results are summarized in Table 1,

dx.doi.org/10.1021/ac502549s | Anal. Chem. 2014, 86, 10992−10995

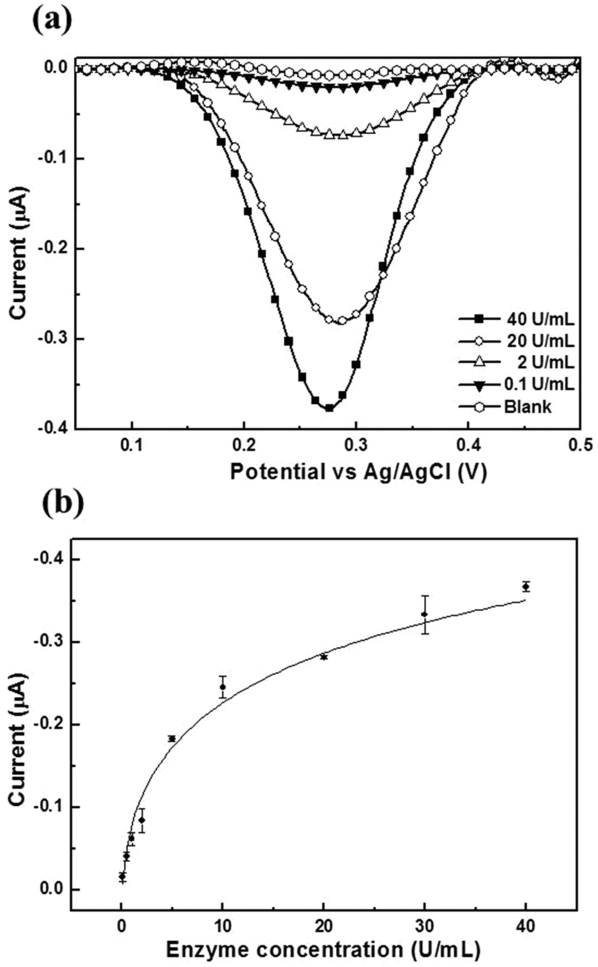


Figure 2. (a) Differential pulse voltammogram for protein kinase A activity on ferrocenylated kemptide substrate with PKA at 40, 30, 20, 10, 5, 2, 1, 0.5, and 0.1 U/mL on the gold electrode modified with the chemosensor. The measurements were carried out with a three-electrode system with Au (working electrode), Ag/AgCl (reference electrode), and Pt (counter electrode) in 0.1 M carbonate buffer (pH 9.2). (b) Plot for the dependence of current response on the concentration of protein kinase A.

Table 1. DPV Measurements for Protein Kinase A Activity on the Ferrocenylated Kemptide in Blood Sample

enzyme added (U/mL)	current expected (A)	current obtained (A)	signal recovery (%)
10	2.32×10^{-7}	2.98×10^{-7}	128.4
5	1.85×10^{-7}	1.59×10^{-7}	85.9
2	7.38×10^{-8}	6.49×10^{-8}	87.8

demonstrating good recovery of signals with respect to the known concentrations of PKA. The slight deviation of the signals from predicted levels occurred presumably because the unused anticoagulant citrate from the blood blocked PKA activity. Nevertheless, these results show that the approach may be versatile enough for the detection of kinase activity in the presence of interfering molecules.

A rapid prototyping technique for microfluidic systems based on a polymer film and double-sided tape was further developed to test single-use and chip-based formats of the electrochemical kinase assay. For this purpose, we used transparent poly(ether sulfone) (PES) films and 3 M double-sided tape. The experimental details and schematic are discussed in the Supporting Information. The PKA detection strategy was similar to the one discussed above. The probe molecule was first immobilized on the working electrode for 12 h. Later, the reaction mixture in the presence of 1× PKA buffer was pumped into the reaction chamber and incubated at 37 °C for 1 h to phosphorylate the substrate. For incubation, the microchip was placed on a hot plate at 37 °C. The phosphorylated product without any filtration was then introduced to the electrochemical cell containing a probe-modified electrode for 30 min. Finally, a DPV analysis was carried out to measure changes in the electrochemical signal following enzymatic catalysis. As expected, the reacted mixtures produced a well-defined electrochemical signal for assays of PKA activity carried out on the polymeric microchip at 50, 10, and 2 U/mL of enzyme as shown in Figure 3.

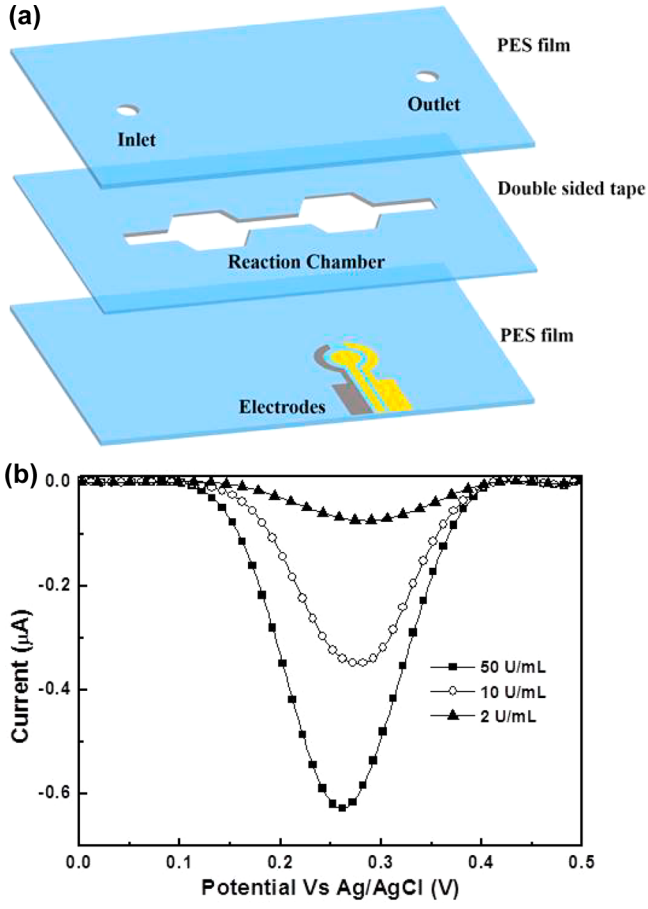


Figure 3. (a) Schematic of polymeric microfluidic device, (b) differential pulse voltammograms for PKA activity on ferrocenylated kemptide substrate with PKA at 50, 10, and 2 U/mL on the thin film gold electrode modified with the synthetic probe. The measurements were carried out with a three-electrode system with Au (working electrode), Ag/AgCl (reference electrode), and Au (counter electrode) in 0.1 M carbonate buffer (pH 9.2).

In conclusion, we report an electrochemical assay for protein kinase activity that employs a synthetic probe immobilized on an electrode surface. The approach involves homogeneous capture of the enzymatic products in the sample solution. It is likely that the proposed method could be easily modified to monitor the activity of other protein kinases with their specific substrates. Importantly, we showed that the synthetic probe

dx.doi.org/10.1021/ac502549s | Anal. Chem. 2014, 86, 10992−10995

specifically bound to the phosphate group of the enzymatic product, resulting in a large increase in voltammetric signals upon enzyme catalysis, even in the presence of citrated blood. This homogeneous assay offers sensitive and reproducible quantification of protein kinase activity. It also showed high fidelity, the versatility to be applied to a human blood test, and the potential for adaptation to chip-based and single-use probe formats. Therefore, the approach described herein is likely to be applied to the high-throughput screening of kinase inhibitors and the diagnosis of diseases caused by irregular kinase activities.

■ ASSOCIATED CONTENT

Supporting Information

Preparation and characterization of probe including ^{1}H, ^{13}C NMR, and HR-MS data; cyclic volammetry diagrams; and detailed experimental procedures for modification of electrode and fabrication of polymeric microchip. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the NRF grant funded by the Ministry of Science, ICT, and Future Planning and by the MOE (Grants NRF-2011-0024786 and 2009-0080734, NRF-2012K1A3A1A04034899)

■ REFERENCES

(1) Blom, N.; Sicheritz-Ponten, T.; Gupta, R.; Gammeltoft, S.; Brunak, S. *Proteomics* **2004**, *4*, 1633.
(2) Cohen, P. *Proc. R. Soc., Ser. B: Biol.* **1988**, *234*, 115.
(3) Tarrant, M. K.; Cole, P. A. *Annu. Rev. Biochem.* **2009**, *78*, 797.
(4) Manning, G.; Whyte, D. B.; Martinez, R.; Hunter, T.; Sudarsanam, S. *Science* **2002**, *298*, 1912.
(5) Cohen, P. *Eur. J. Biochem.* **2001**, *268*, 5001.
(6) Schroder, K.; Hertzog, P. J.; Ravasi, T.; Hume, D. A. *J. Leukocyte Biol.* **2004**, *75*, 163.
(7) Tedgui, A.; Mallat, Z. *Physiol. Rev.* **2006**, *86*, 515.
(8) Witt, J. J.; Roskoski, R. *Anal. Biochem.* **1975**, *66*, 253.
(9) Jensen, K. F.; Houlberg, U.; Nygaard, P. *Anal. Biochem.* **1979**, *98*, 254.
(10) Geahlen, R. L.; Anostario, M.; Low, P. S.; Harrison, M. L. *Anal. Biochem.* **1986**, *153*, 151.
(11) Houseman, B. T.; Huh, J. H.; Kron, S. J.; Mrksich, M. *Nat. Biotechnol.* **2002**, *20*, 270.
(12) Mathis, G. *Clin. Chem.* **1995**, *41*, 1391.
(13) Seethalal, R.; Menzel, R. *Anal. Biochem.* **1997**, *253*, 210.
(14) Hertzberg, R. P.; Pope, A. J. *Curr. Opin. Chem. Biol.* **2000**, *4*, 445.
(15) Rhee, H. W.; Lee, S. H.; Shin, I. S.; Choi, S. J.; Park, H. H.; Han, K.; Park, T. H.; Hong, J. I. *Angew. Chem., Int. Ed.* **2010**, *49*, 4919.
(16) Lee, S. H.; Rhee, H. W.; van Noort, D.; Lee, H. J.; Park, H. H.; Shin, I. S.; Hong, J. I.; Park, T. H. *Biosens. Bioelectron.* **2014**, *57*, 1.
(17) Kerman, K.; Chikae, M.; Yamamura, S.; Tamiya, E. *Anal. Chim. Acta* **2007**, *588*, 26.
(18) Kerman, K.; Song, H.; Duncan, J. S.; Litchfield, D. W.; Kraatz, H. B. *Anal. Chem.* **2008**, *80*, 9395.
(19) Song, H. F.; Kerman, K.; Kraatz, H. B. *Chem. Commun.* **2008**, 502.
(20) Lee, D. H.; Im, J. H.; Son, S. U.; Chung, Y. K.; Hong, J. I. *J. Am. Chem. Soc.* **2003**, *125*, 7752.
(21) Shin, I. S.; Bae, S. W.; Kim, H.; Hong, J. I. *Anal. Chem.* **2010**, *82*, 8259.
(22) Kim, S. K.; Lee, D. H.; Hong, J. I.; Yoon, J. *Acc. Chem. Res.* **2009**, *42*, 23.
(23) Liu, G.; Choi, K. Y.; Bhirde, A.; Swierczewska, M.; Yin, J.; Lee, S. W.; Park, J. H.; Hong, J. I.; Xie, J.; Niu, G.; Kiesewetter, D. O.; Lee, S.; Chen, X. Y. *Angew. Chem., Int. Ed.* **2012**, *51*, 445.