TiO_2-assisted silver enhanced biosensor for kinase activity profiling†

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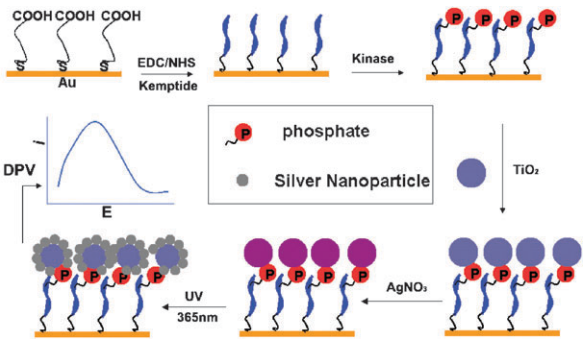
An electrochemical biosensor has been developed for sensitive determination of protein kinase activity and inhibition, taking advantage of specific binding of the phosphate groups with TiO_2 nanoparticles, on which silver nanoparticles are photo-catalytically deposited to achieve signal enhancement.

Protein kinase-catalyzed phosphorylation plays a crucial role in signal transduction pathways that regulate many cellular biological processes, including apoptosis, cell growth, and differentiation in response to extracellular stimuli.[1] Thus, to better understand the fundamental biochemical processes at molecular level and find further application in drug discovery, the exploration of kinase activity and its inhibition by potential inhibitors is indispensable. Up to now, most commonly used tools to perform kinase activity screening are radioactive methods,[2] fluorescently-labeled phosphor-specific antibody surface plasmon resonance[3–5] and fluorescence resonance energy transfer-based systems.[6–10] Recently, kinase-catalyzed phosphorylation with ATP analogs containing biotin, thiol and ferrocene conjugates has become an alternative to radioactive methods for kinase activity detection and characterization.[11–14] In order to eliminate the additional modification of the peptides, ATP and other related molecules, label-free methods are attractive for quantitatively monitoring kinase activity. Electrochemistry is one of the promising approaches to address such criteria due to its sensitivity and simplicity. Kerman and co-workers[15] developed a label-free approach for the quantitative detection of peptide tyrosine phosphorylation using the electrochemical oxidation current signal of tyrosine. Willner and co-workers[16] reported a novel method for casein kinase by the voltammetric response of silver ions associated with the phosphorylated product. Currently, metal oxides such as TiO_2 have been used to selectively isolate phosphopeptides, where the phosphate functional groups can bind to the surface of TiO_2 particles.[17,18] On the other hand, TiO_2 can act as a sensitizer for light-induced redox processes due to its electronic structure characterized by a filled valence band and an accessible empty conduction band,[19] being applied in solar cells[20] or photocatalytic reactions.[21,22] Under UV-irradiation, Ag^+ ions could be photo-catalytically reduced to Ag nanoparticles by the excited electrons and subsequently deposited onto the TiO_2 surface.[23,24]

Based on these principles, herein we introduce a smart sensing protocol based on multi-nanoparticle deposition through a signal transforming medium for profiling kinase activity and its inhibition. It is anticipated that the deposited Ag nanoparticles can be used as targets and further oxidized to Ag^+ ions during the electrochemical measurement. Taking advantage of the specific binding of TiO_2 nanoparticles and the enhancement of silver nanoparticles, this biosensor shows high sensitivity. The scaffold not only provides a facile readout of protein kinase activity, but also offers a possible way to explore the throughput screening of inhibitors.

To realize this purpose, protein kinase A (PKA) was employed as the model enzyme and Kemptide (H-LRRASLG-OH) as the designed peptide. The kinase activity assay procedure is summarized in Scheme 1. First, Kemptide was immobilized on the gold electrode surface based on the activation with mercaptoacetic acid (MAA) and *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC)–*N*-hydroxysuccinimide (NHS) (see ESI†).[25] After the PKA-catalyzed reaction in the presence of ATP, the phosphate groups were transferred to the serine residues of Kemptides on the electrode surface. The surface characteristics were recorded by FT-IR. The peaks at 923.80 cm^{-1} and 1268.23 cm^{-1} were attributed to phosphates and peptides respectively (ESI, Fig. SI-1†). Then a TiO_2 suspension was dropped on to the modified electrode surface for 30 min, followed by a washing step with an elution solution of 2,5-dihydroxybenzoic acid (DHB) which was capable of specifically displacing non-specifically bound acidic peptides from the TiO_2. Subsequently, the phosphorylated peptides



Scheme 1 Schematic illustration of the electrochemical biosensor for the detection of kinase-catalyzed phosphorylation in the presence of ATP by silver enhancer photocatalytically deposited on TiO_2 nanoparticles attached with phosphate groups. The substrate peptide (Kemptide) is immobilized on the surface of a gold electrode. Protein kinase A (PKA)-catalyzed reaction transfers the phosphate group to the serine residue of Kemptide. The phosphate group is specifically linked with TiO_2 nanoparticle and silver nanoparticle enhancement by photocatalytic reaction. Silver as the detected target is oxidized by DPV.

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were specifically linked by TiO_2 nanoparticles for signal transformation.[17,18] Considering Ag^+ ions could be photocatalytically reduced to Ag nanoparticles by the excited electrons of titanium oxides under UV light,[23,24] the modified electrode was immersed into the $AgNO_3$ solution (0.5 M, water–methanol) for 10 min, and then washed with water and dried with purified N_2. Finally, the electrode surface was irradiated under a UV light at 365 nm with a drop of water–methanol solution. The deposited silver nanoparticles acting as the detector targets could be analyzed by differential pulse voltammetry (DPV). The electrochemical signal produced is statistically proportional to the quantity of the phosphorylated peptides, which is related to the kinase activity. In order to avoid the excess silver interfering with the response signals, the electrode was washed with a sodium thiosulfate fixer solution that converted the silver cations into $Ag(S_2O_3)_3^{5-}$ anions before electrochemical detection. It has to be noted that the silver solution should be prepared immediately before application and the whole silver deposition procedure should be carried out in a dark room. To verify the multi-deposition based sensing concept, we synthesized the multi-metal nanoparticles according to this procedure. An aqueous solution of silver nitrate at 1 M was uniformly mixed with TiO_2 suspension solution (4 mg mL^{-1}) for 5 min. After being centrifuged and washed with water, the mixture was irradiated under UV light at 365 nm in the ethanol–water solution. The color of the suspension was changed from white to brownish-gray immediately, due to surface plasmon resonance of the deposited Ag nanoparticles. The TiO_2@Ag nanoparticles obtained were characterized by transmission electron microscopy, as shown in Fig. SI-2.† We could see that silver nanoparticles with diameters of 2–5 nm were formed on the surface of the TiO_2 particles, which could induce enhancement of the response signal, and indicate that the proposed protocol was feasible.

The silver deposition time with regard to the response currents was investigated since the deposition step is necessary for signal detection. By stopping the UV irradiation at different time intervals, the current response was measured (ESI, Fig. SI-3†). When the silver deposition time was above 10 min, the current response starts to remain stable. Additionally, the full surface coverage concentration of Kemptide at 350 μM incubated on the electrode surface for 5 hours was employed to obtain optimal results (ESI, Fig. SI-4†). The activated Kemptides were reacted with different concentrations of PKA and the response of the deposited silver was measured by DPV as shown in Fig. 1 inset. When the PKA units increased gradually, the electrochemical signals increased accordingly. The dependence of the response on the PKA concentration is shown in Fig. 1. The bioactive unit of PKA was detected down to a limit of 0.2 U mL^{-1} with a facile readout, being more sensitive than or comparable to the ones in previous reports.[13,14] The linear range was from 0 to 1 U mL^{-1}, and PKA concentration at 5 U mL^{-1} gave the maximum response at saturation with a relative standard deviation of 8.2% (n = 5). Thus, 5 U mL^{-1} PKA was employed in the following phosphorylation reaction and kinase inhibition assay. Moreover, the biosensor exhibited good specificity for profiling kinase activity. In order to prove

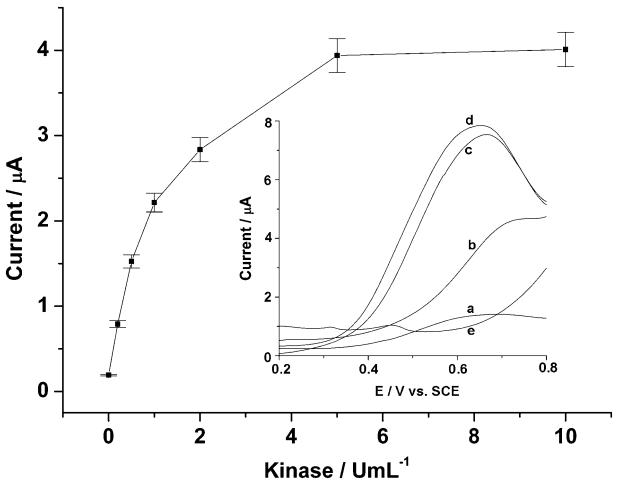

Fig. 1 Plot for the dependence of current response on the concentration of protein kinase A. Inset: electrochemical differential pulse voltammetry measurements for kinase activity on Kemptide substrate with PKA at (a) 0 U mL^{-1}, (b) 0.2 U mL^{-1}, (c) 5 U mL^{-1}, (d) 10 U mL^{-1}, (e) PKA at 5 U mL^{-1} on the gold electrode only functionalized with MAA without Kemptide as comparison. The reaction mixture containing 150 μM ATP, 0.1 mg mL^{-1} BSA and 0.2% β-mercaptoethanol in the kinase buffer were incubated on the modified electrode surface for 60 min at 30 °C in a humidified chamber. DPV measurements were performed with a three-electrode system; the reference electrode was separated from the working solution by a double electrolytic salt bridge. DPV determination was performed in 0.1 mol L^{-1} HAc–NaAc buffer solutions (pH 5.2) from +0.10 to +0.80 V (*versus* SCE).

the selectivity of the signal transfer, the electrode was only modified by MAA without Kemptide for investigation. As expected, no significant electrochemical response was recorded in Fig. 1 inset, line e.

The proposed TiO_2-assisted silver enhanced biosensing protocol for kinase activity assay could be used to quantitatively characterize the inhibition of kinase in the presence of small molecule inhibitors. Inhibition of PKA phosphorylation activity was tested by comparing signals from samples incubated with PKA inhibitors, and the half-maximal inhibition value IC_{50} was detected. To investigate its quantitative inhibition assay, ellagic acid (4,4′,5,5′,6,6′ hexahydroxydiphenic acid 2,6,2′,6′-dilactone) was employed as an model inhibitor. As a cell-permeable and potent antioxidant, ellagic acid has anti-mutagenic and anti-carcinogenic properties.[26] As expected, in the presence of the ellagic acid, the DPV signals decreased with increasing efficiency of the inhibitor. Only a weak electrochemical current was observed with the addition of ellagic acid at 8 μM as shown in Fig. 2 inset. The inhibition of PKA activity on Kemptide using ellagic acid was monitored and an IC_{50} was measured to be about 5.5 μM from Fig. 2. These results confirm that the TiO_2–Ag nanoparticle based electrochemical format has the potential for screening PKA inhibitors as well as determining the IC_{50} values.

In conclusion, a novel TiO_2-assisted silver nanoparticle enhanced biosensor has been developed for kinase activity and inhibition assay. The results indicate that the method is highly sensitive with a low detection limit. A well-known kinase system is employed here to establish and verify this

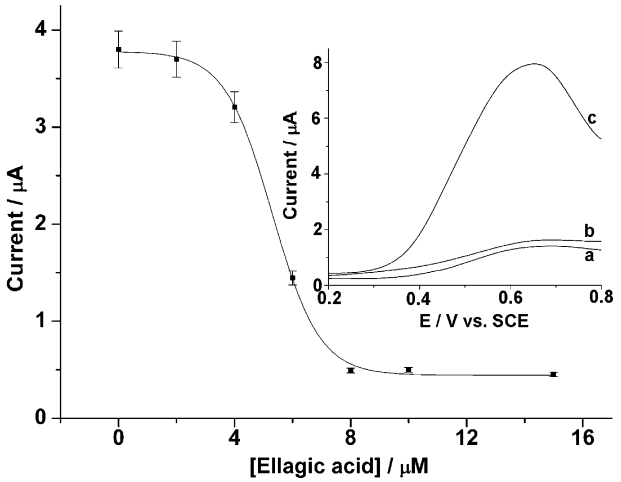

Fig. 2 Plot for the dependence of current response on the concentration of kinase inhibitor (ellagic acid). Inset: electrochemical differential pulse voltammetry measurements for kinase activity in the presence of ellagic acid (b) 8 μM, (c) 0 μM with 5 U mL^{-1} PKA, and (a) with 0 U mL^{-1} PKA on a 350 μM Kemptide immobilized gold electrode. The reaction conditions are the same as in Fig. 1.

format, which includes the phosphorylation process followed by the enhancement of the phosphorylated products by the specific deposition of TiO_2 and silver nanoparticles for the first time. It is a general approach which can be readily transferable to the other substrates kinases, and is promising for high throughput screening of inhibitors as potential therapeutic agents.

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