Electrochemical detection of kinase-catalyzed thiophosphorylation using gold nanoparticles†

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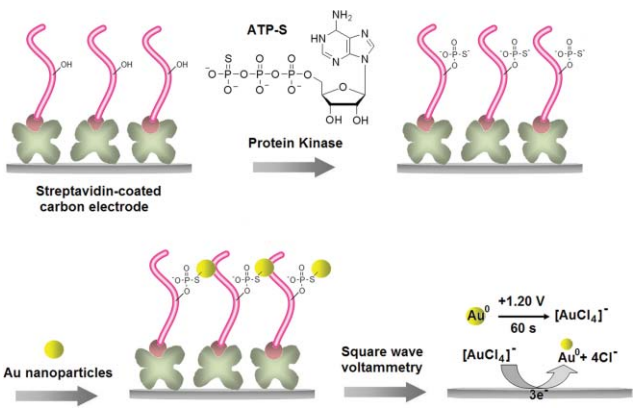
An electrochemical biosensor for kinase-catalyzed reactions is coupled with the thiophosphorylation of the substrate peptide using adenosine 5′-[γ-thio] triphosphate (ATP-S) as the co-substrate.

Protein kinases have a significant role at the crossroads of the cellular communication network, and function as master switches. During phosphorylation, the phosphoryl group at the γ-position of ATP is transferred to specific serine, threonine, or tyrosine residues of the protein.[1] Upon phosphorylation, the abnormally enhanced signals emanating from protein kinases turn these enzymes into oncoproteins and this results in the malfunction of cellular signalling networks leading to cancers and other proliferative diseases.[2] Recently, Mann and co-workers[3] reviewed the approaches that focus on the design of ATP analogues and protein kinase binding site mutants. Adenosine 5′-[γ-biotin] triphosphate (ATP-biotin) has been utilized in phosphorylation assays as a promising alternative to the existing technologies.[4] Brust and co-workers[5] utilized the optical properties of Au nanoparticles and developed kinase activity assays using ATP-biotin. Recently, Shokat and co-workers[6] have utilized ATP-S to create an epitope for thiophosphate-ester specific antibodies.

The tools routinely used to detect phosphorylation involve mass spectroscopy,[7] radioisotopes[8] and fluorescent labels,[9] phospho-specific antibodies in connection with surface plasmon resonance (SPR)[10] and fluorescence resonance energy transfer (FRET)[11]-based systems. Herein, the proof-of-concept study of an electrochemical biosensor is presented for the detection of kinase activity using protein kinase C (PKC) as the model enzyme. For the first time, the method is based on the labelling of a specific phosphorylation reaction with ATP-S and Au nanoparticles, followed by electrochemical detection using the chloride redox chemistry on Au nanoparticles. The experimental conditions, such as ATP-S and PKC concentration, reaction time and temperature, were optimized for thiophosphorylation reactions. The biotinylated protein kinase Cζ substrate peptide (Biotin-SIYRRG*S*RRWRKL) was immobilized on a streptavidin-coated screen-printed carbon electrode (SPCE) surface (see ESI†). The phosphorylation reaction was coupled with the thiolation of the substrate peptide using adenosine 5′-[γ-thio] triphosphate (ATP-S) as the co-substrate. When the thiophosphorylated substrate peptide was exposed to Au nanoparticles (5 nm, A_{525} = 0.5), the affinity between the thiophosphate and Au nanoparticles resulted in the attachment of Au nanoparticles on the substrate peptide (Scheme 1). The electrochemical reduction response obtained from the chloride ions on Au nanoparticles made it possible to monitor the activity of PKC *in vitro*.

The voltammetric detection of Au nanoparticles *via* a Cl^- chemistry on a single surface has recently been reported as a simple and highly sensitive detection method.[12] Single-surface electrochemical detection involved the oxidation of the Au nanoparticles at 1.2 V for 1 min in 0.1 M HCl (pH 1.2). As a result of this oxidation step in a highly acidic environment, $[AuCl_4]^-$ ions were produced and the biomolecules were oxidized and denatured on the surface. The denaturation of the biomolecules allows an open surface for the $[AuCl_4]^-$ ions to be reduced at ~0.4 V (*vs.* Ag/AgCl inner reference electrode of the SPCE). Fig. 1 shows the voltammetric responses obtained from the reduction scans following the PKC-catalyzed thiophosphorylation reactions. The concentrations of substrate peptide and ATP-S were kept constant at 50 μM and 100 μM, respectively, and the dependence of the current responses on PKC concentration was recorded as shown in Fig. 1a, b and c. The peak current heights reached a saturation level when PKC concentrations over 100 U mL^{-1} were used. The dependence of peak current responses on PKC concentration was



Scheme 1 Schematic illustration of the electrochemical principle for the detection of kinase-catalyzed thiophosphorylation using adenosine 5′-[γ-thio] triphosphate (ATP-S) and Au nanoparticles. The biotinylated substrate peptide is immobilized on the surface of the streptavidin-coated screen-printed carbon electrode (SPCE). Protein kinase C (PKC)-catalyzed reaction transfers a thiophosphate group to the serine residue of the peptide. The incubation of the thiophosphorylated peptide with Au nanoparticles causes the attachment of Au nanoparticles on the surface. The surface-attached Au nanoparticles are processed through a chloride-based redox chemistry using square wave voltammetry (SWV).

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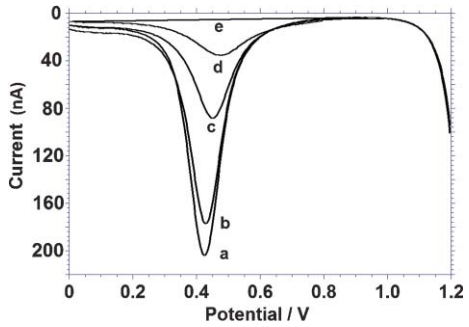

Fig. 1 Square-wave voltammograms of Au nanoparticles on SPCEs modified with 50 μM substrate peptide in the presence of increasing concentrations of protein kinase C (PKC) at (a) 100 U mL^{-1}, (b) 50 U mL^{-1}, (c) 10 U mL^{-1} with 100 μM ATP-S, (d) 100 U mL^{-1} PKC with 10 μM ATP-S, (e) no substrate peptide was immobilized on the streptavidin-coated SPCE in the presence of 75 μM ATP-S and 100 U mL^{-1} PKC. Kinase assay buffer included 20 mM Tris, 0.5 mM EDTA, 10 mM $MgCl_2$, 500 μg mL^{-1} phosphatidyl serine (pH 7.5). SWV measurements were taken at a frequency of 15 Hz in 0.1 M HCl (pH 1.2).

plotted (Fig. 2A). However, the current responses were also dependent on ATP-S concentration. When 10 μM ATP-S was used together with 100 U mL^{-1} PKC, a small current response was observed (Fig. 1-d), indicating that the surface-immobilized substrate peptides were not thiophosphorylated efficiently in the presence of 10 μM ATP-S. To demonstrate the specificity of the thiophosphorylation reactions, we prepared SPCEs modified with streptavidin only. As expected, streptavidin was not modified by PKC and no significant electrochemical responses were recorded (Fig. 1-e). The detection limit for PKC was determined as 10 U mL^{-1} with a linear range up to 50 U mL^{-1}.

For the optimization of experimental conditions, a series of measurements were taken in the presence of varying ATP-S concentrations and 100 U mL^{-1} PKC using the same assay conditions (Fig. 2B). As the concentration of ATP-S increased, the thiophosphorylation of the peptides resulted in the attachment of Au nanoparticles on the surface. The current responses remained the same for concentrations over 75 μM. Thus, 75 μM ATP-S was applied for further kinase assays. When no ATP-S was used in the assay buffer, we obtained no significant current response, indicating the suppression of non-specific adsorption of Au nanoparticles on the electrode surface by the stringent washing of the SPCEs.

Au nanoparticles were imaged using scanning electron microscopy (SEM) (Fig. 3) and transmission electron microscopy (TEM,

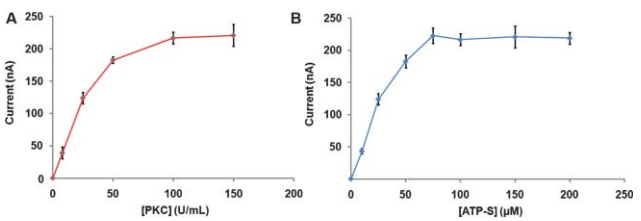

Fig. 2 Plots for the dependence of current responses on PKC (A) and ATP-S concentration (B) for the PKC-catalyzed thiophosphorylation reactions. Error bars indicate the standard deviation of three measurements (n = 3). Other conditions were as described in Fig. 1.

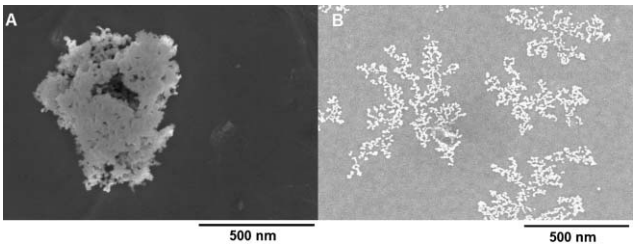

Fig. 3 (A) Scanning electron microscope (SEM) image of Au nanoparticles after the kinase-catalyzed thiophosphorylation reaction in solution using 75 μM ATP-S and 100 U mL^{-1} PKC. Au nanoparticles formed large aggregates as a result of the affinity towards the peptides. (B) SEM image of Au nanoparticles after thiophosphorylation reaction using 75 μM ATP and 100 U mL^{-1} PKC. The absence of thiophosphate groups in the reaction suppressed the formation of large aggregates.

ESI† Fig. 1) following the thiophosphorylation reactions in solution. ATP-S and ATP were used as the co-substrates in separate reactions and aliquots of the assay solutions were pipetted on solid substrates for imaging. In the presence of ATP-S, the peptides were thiophosphorylated and the affinity between the peptides and Au nanoparticles was substantially increased, resulting in the formation of large aggregates (Fig. 3A). However, when only ATP with no γ-thiophosphate substitution was used, we could not observe the formation of large aggregates in comparison with the ones formed in the presence of ATP-S (Fig. 3B).

The reaction displays significant temperature dependence as shown in Fig. 4. The optimum response was obtained after the incubation of the electrodes at 30 °C. When the electrodes were incubated at 45 °C (Fig. 4b) and 4 °C (Fig. 4c), no significant current responses were obtained. Fig. 5A shows the current responses obtained at various temperatures with the error bars indicating the standard deviation of three measurements (n = 3).

The time dependence of the thiophosphorylation was determined by stopping the reaction at different time intervals and measuring the current response (Fig. 5B). A saturation level in the signals was reached after 1 h incubation in the presence of 100 U mL^{-1} PKC. When the reaction was stopped at 90 min, no significant increase in the reduction peak responses was recorded. The dependence of responses on the incubation time with Au

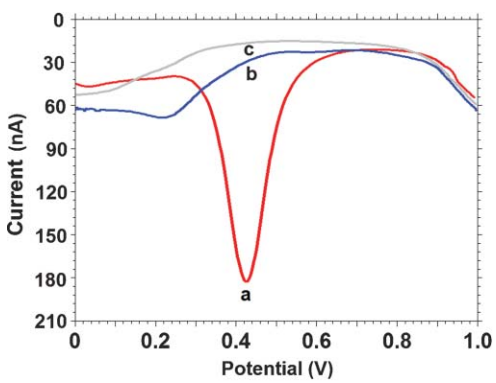

Fig. 4 Square wave voltammograms of Au nanoparticles on SPCEs modified with 50 μM substrate peptide following the kinase reactions in the presence of 75 μM ATP and 100 U mL^{-1} PKC at (a) 30 °C, (b) 45 °C, (c) 4 °C.

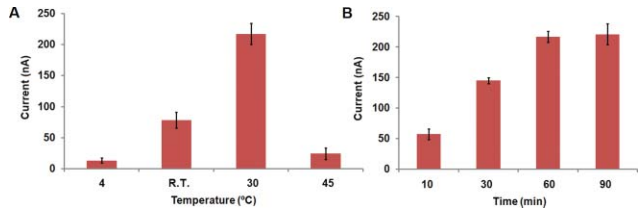

Fig. 5 Plots for the dependence of current responses on temperature (A) and incubation time (B) for the PKC-catalyzed thiophosphorylation reactions. Error bars indicate the standard deviation of three measurements (n = 3). Other conditions were as described in Fig. 1.

nanoparticles was monitored (data not shown). The reduction signals increased gradually until 1 h and then remained stable. Thus, 1 h of incubation time with Au nanoparticles was employed following the PKC-catalyzed reactions using ATP-S.

The proof-of-concept studies are demonstrated for the use of ATP-S in kinase-catalyzed reactions on the surface-immobilized substrate peptides. The experimental conditions were optimized using Au nanoparticles as the electro-active indicator. The chloride-based redox chemistry on a single surface was utilized to detect PKC activity. The electrochemical biosensor reported here is promising for further development toward *in vitro* electrochemical kinase profiling studies. We anticipate that biosensors will have important implications for the development of high-throughput and multiplexed nanoparticle-based kinase activity assays for the diagnosis of cancers and the screening of kinase inhibitors as promising anti-cancer drugs.

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