Electrochemical detection of kinase-catalyzed phosphorylation using ferrocene-conjugated ATP†

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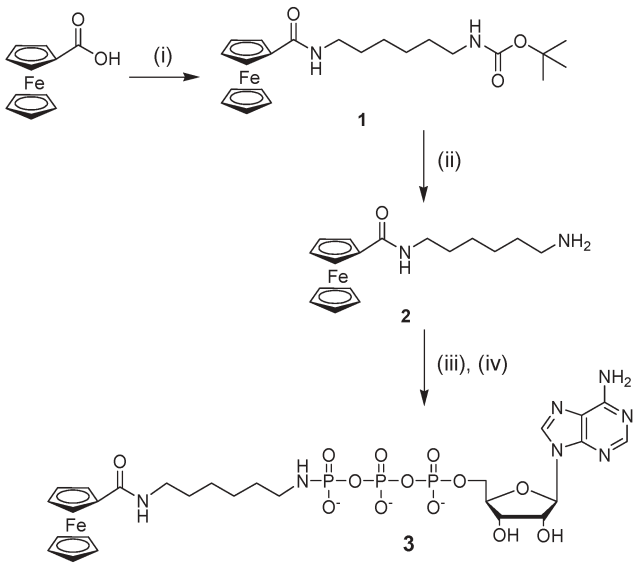
Adenosine-5′-[γ-ferrocene] triphosphate is exploited as a co-substrate for the phosphorylation of the surface-immobilized peptide C-SIYRRGSRRWRKL by protein kinase C, in which the γ-ferrocene phosphate is transferred to the peptide and then detected by cyclic voltammetry.

In the cellular communication network, many enzymes and receptors are switched "on" or "off" in other terms "phosphorylated" and "dephosphorylated" by the protein kinases. During phosphorylation, a phosphoryl group from ATP is transferred to specific serine, threonine, or tyrosine residue of the protein. As a result of these modifications, the function or localization of the protein may change, which in some cases may lead to the formation of the oncoproteins.[1] Abnormal protein phosphorylation is a cause of major diseases, including cancer, diabetes and chronic inflammatory diseases.[2] Analytical methods to quantify protein kinase activity are critical for understanding their role in the diagnosis and therapy of these diseases. Current methods for the detection of protein phosphorylation rely on radio-labeled ATP,[3] fluorescence-based methods,[4] and fluorescence resonance energy transfer (FRET).[5] Recently, ATP analogs containing biotin and thiol conjugations have been exploited for the detection of phosphorylation reactions.[6] However, additional modification of the peptides with an electro-active or optical label is necessary, which increases the cost and causes tedious and time-consuming handling procedures. In this communication, we demonstrate the use of an alternative detection method making use of the electro-active adenosine-5′-[γ-ferrocene] triphosphate (Fc-ATP, **3**), as the co-substrate for protein kinase C (PKC).

The procedure for the preparation of the Fc-ATP conjugate **3** is summarized in Scheme 1. Ferrocenecarboxylic acid was reacted with *t*-butyl-6-aminohexylcarbamate in the presence of dicyclohexylcarbodiimide (DCC) to give Boc-protected conjugates **1** in good yield, following the deprotection of the Boc to give free amine **2**. After ion exchange, the triethylammonium salt of ATP was converted into its trimetaphosphate form in DMF in the presence of DCC and the solution was added to a solution of the free amine **2**, resulting in the formation of the desired compound **3** (Fc-ATP). The product was characterized spectroscopically using ^{1}H, ^{31}P NMR spectroscopy and MS. In the ^{31}P NMR, compound **3** exhibited three signals, with a doublet for the γ-P at δ −0.07 (*vs.* H_3PO_4) indicating formation of a phosphoramide bond at the γ position (ESI,† Fig. S1).[7]



Scheme 1 Preparation of the Fc-ATP conjugate **3**. *Reagents and conditions*: (i) TEA, HBTU, $BocNH(CH_2)_6NH_2$; (ii) (a) TFA, DCM, (b) TEA; (iii) ATP, DCC, DMF; (iv) compound **2**, MeOH.

TOF-MS of Fc-ATP was recorded in acetonitrile–water solution with a core voltage of 135 V and resulted in the detection of a peak with *m*/*z* of 818.2 as would be expected for the 4H adduct (ESI,† Fig. S2).

The electrochemical approach was implemented using a well-defined kinase system on screen-printed gold electrodes (SPEs, ESI,† Fig. S3). A schematic illustration of the electrochemical principal for the detection of kinase-catalyzed phosphorylation using Fc-ATP as the co-substrate is shown in Scheme 2. The substrate peptide is immobilized on the surface of the SPE *via* a sulfur bond. Protein kinase C (PKC)-catalyzed reaction transfers a γ-phosphate-Fc group to the serine residue of the peptide. The Fc group attached to the peptide is electrochemically observed using cyclic voltammetry (CV).

Using the surface-immobilized peptides, the current density responses were recorded in the presence and absence of PKC in the assay solution as shown in Fig. 3. The CV response in Fig. 1(a) shows the similar redox behaviour of Fc-ATP with that observed in solution, however, the peak potentials were slightly shifted to higher values (ESI,† Fig. S4(A) and (B)) indicating the presence of a layer (peptide film) on the surface, which hampered the redox process to occur at a lower potential. The oxidation peak was

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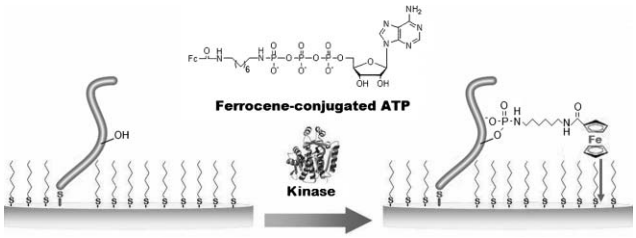

Scheme 2 Schematic illustration of the electrochemical principle for the detection of kinase-catalyzed phosphorylation using Ferrocene-conjugated ATP (Fc-ATP). The substrate peptide is immobilized on the surface of the screen-printed gold electrode (SPE). Protein kinase C (PKC)-catalyzed reaction transfers a γ-phosphate-Fc group to the serine residue of the peptide. The surface-attached Fc groups are detected *via* electrochemical techniques (cyclic voltammetry, CV and square wave voltammetry, SWV).

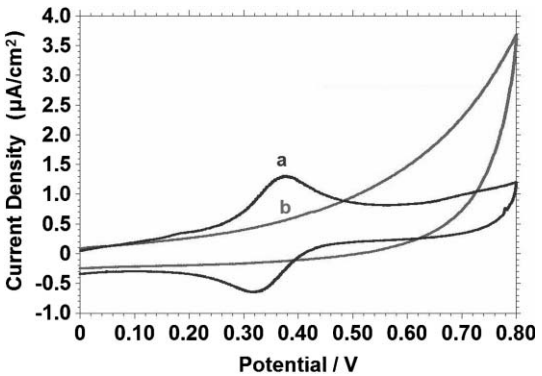

Fig. 1 Cyclic voltammograms for the PKC-catalyzed phosphorylation reactions (a) in the presence of 10 U mL^{-1} PKC with 100 μM Fc-ATP, (b) in the absence of PKC. Kinase assay solution also included 20 mM Tris, 0.5 mM EDTA, 10 mM $MgCl_2$, 500 μg mL^{-1} phosphatidyl serine (pH 7.5). Measurements were taken in 0.1 M $NaClO_4$ solution at 100 mV s^{-1}.

detected at ~0.32 V and the reduction peak was observed at ~0.38 V (*vs*. Ag/AgCl inner reference electrode). The separation of the redox peak potentials indicated that one electron was involved in the process. This electrochemical behaviour was expected from the well-defined electrochemical properties of Fc. The absence of any redox current signals in Fig. 1(b) indicated that the attachment of Fc-ATP to the peptides were dependent on the presence of the kinase. Moreover, no redox activity in the absence of PKC showed the successful suppression of the non-specific adsorption of Fc-ATP on the electrode surface.

SWV was also applied to detect the Fc oxidation current signals at low concentrations of PKC as shown in ESI,† Fig. S5. The substrate peptide and Fc-ATP concentration was kept constant at 200 and 100 μM, respectively. ESI,† Fig. S5(a) shows the current response obtained in the presence of 0.1 U mL^{-1} PKC. The increasing trend of the current density responses were recorded, as the concentration of PKC increased (Fig. 2).

For the optimization of experimental conditions, a series of measurements were taken in the presence of varying Fc-ATP concentrations and 100 U mL^{-1} PKC using the same assay conditions. As the concentration of Fc-ATP increased, the phosphorylation of the peptides resulted in the high current responses on the surface (ESI,† Fig. S6). The current responses remained the same for concentrations over 100 μM. Thus, 100 μM

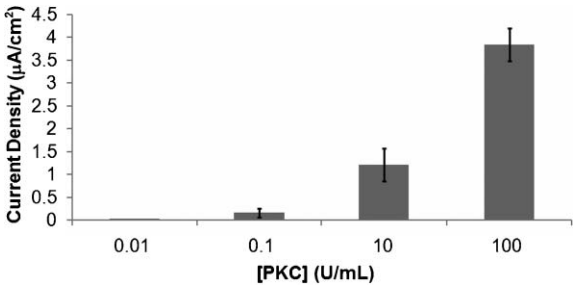

Fig. 2 The dependence of current density responses on the concentration of PKC obtained from the cyclic voltammetric data. The error bars indicate the standard deviation (n = 3). Other experimental conditions were as described in Fig. 1.

Fc-ATP was applied for further kinase assays. When no ATP-F was used in the assay buffer, we obtained no significant current response indicating the suppression of non-specific adsorption of Fc-ATP on the electrode surface by the stringent washing of the SPEs as described in the experimental. When low concentrations of Fc-ATP were used, no current responses were observed. The dependence of Fc-ATP concentration on the current density signals is plotted in Fig. 3.

The dependence of incubation time was monitored for the optimization of Fc-ATP responses. The concentrations of substrate peptide, PKC and Fc-ATP were kept constant at 200 μM, 100 U mL^{-1} and 100 μM, respectively, and the dependence of the current responses on incubation time at 30 °C was recorded as shown in Fig. 4. The peak current heights reached a saturation level, when the assay solution was incubated for 1 h. When the kinase reaction was allowed to continue only for 20 min, a small current response was observed indicating that the surface-immobilized substrate peptides were not phosphorylated efficiently in the presence of 100 μM Fc-ATP (ESI,† Fig. S7).

The electrochemical approach described enables us to carry out activity profiling of kinases. Moreover, the approach is readily versatile for the detection of the kinase activity in the presence of small molecule inhibitors, which hold great promise in cancer therapy. The results of our research are expected to have a major impact on our understanding of the fundamental process of protein phosphorylation and will undoubtedly shed light on the kinase activity and how the activity is affected and ultimately modulated by the presence of alternative substrates, inhibitors and

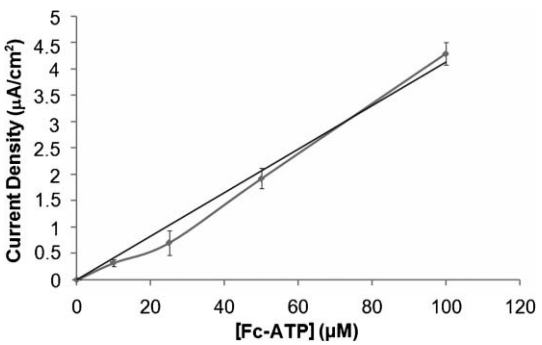

Fig. 3 Plot for the dependence of current density responses on the concentration of Fc-ATP. Other experimental conditions were as described in Fig. 1.

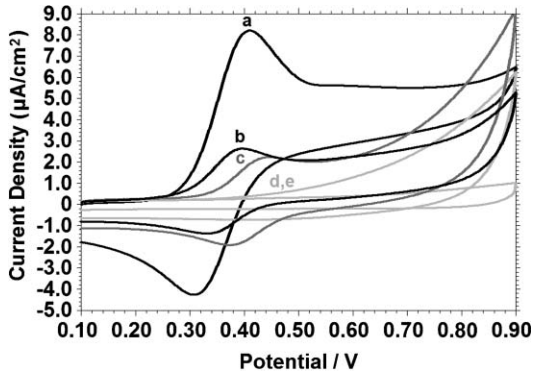

Fig. 4 Cyclic voltammograms for the PKC-catalyzed phosphorylation reactions in the presence of 100 μM Fc-ATP and 100 U mL^{-1} PKC at varying incubation periods at 30 °C, (a) 1 h, (b) 20 min, (c) 10 min, (d) 5 min and the electrodes were not incubated in the assay solution (e). Other experimental conditions were as described in Fig. 1.

other kinases. Future work in our laboratory will provide key data about kinase-catalyzed phosphorylation reactions *in vitro* and inhibitor activities. We anticipate that the miniaturized electrochemical biodevices will have important implications for the drug discovery.

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