

Electronic Supplementary Information (ESI) for

**Electrochemical detection of
kinase-catalysed phosphorylation
using ferrocene-conjugated ATP**

Haifeng Song, Kagan Kerman and Heinz-Bernhard Kraatz*

Department of Chemistry, University of Western Ontario,
1151 Richmond Street, London, ON, N6A 5B7, Canada

*Corresponding author: hkraatz@uwo.ca

EXPERIMENTAL

Reagents

All synthesis reactions were carried out under an atmosphere of argon unless indicated otherwise. Diethylaminoethyl (DEAE)-cellulose, adenosine 5'-triphosphate (ATP) disodium salt was obtained from Sigma and used as received. Dowex AG 50W-X8 was obtained from Bio-Rad Laboratories (Ontario, Canada). N,N'-Dicyclohexylcarbodiimide (DCC), *O*-(1H-Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) was obtained from AdvancedChemTech (KY, USA). Dimethylformamide (DMF) and dichloromethane (DCM) was distilled from CaH₂ before use. Methanol was distilled from magnesium turning with the presence of iodine. Ferrocenecarboxylic acid¹ and *t*-butyl-6-aminohexylcarbamate² were prepared according to the literature procedures.

The protein kinase C_ζ peptide (SIYRRGSRWRKL) was purchased from Calbiochem (EMD Biosciences, USA) and modified with a cysteine residue at N-terminal in our laboratory. The modified protein kinase C_ζ pseudosubstrate sequence contains Ser₁₁₉ instead of Ala₁₁₉.³ Protein kinase C from rat brain (E. C. 2.7.1.37) was purchased from Sigma in 50% glycerol containing 20 mM Tris, 0.5 mM EDTA, 0.5 mM EGTA, 5 mM DTT, 100 mM NaCl, 0.02% Tween 20, and 1 μg/mL leupeptin. One unit (U) of PKC will transfer 1 nanomole of phosphate from ATP into histone H1 per min at 30°C.^{4,5} Adenosine 5'-[γ-Ferrocene] triphosphate (Fc-ATP) was synthesized in our laboratory. Other reagents were purchased from Merck. All solutions were prepared and diluted using ultra-pure water (18.3 MΩ-cm) from the Millipore Milli Q system.

Instruments

Cyclic voltammetry (CV) was performed using an CHInstruments 660 system (Austin, TX). DEP-chips as screen-printed gold electrodes (SPEs) were kindly donated by BioDevice

Technology Ltd. (Ishikawa, Japan). The total length of a SPE was 11 mm, and the geometric area of the working electrode was 2.64 mm². ¹H, ¹³C, ³¹P NMR experiments were performed on a Bruker Avance 500 MHz spectrometer and chemical shifts were referenced to the residue DMSO (2.50ppm for ¹H and 39.52ppm for ¹³C) and H₂O (4.79ppm). Mass spectrometry was carried out using a Perkin Elmer-Sciex API 365 instrument.

Synthesis of Fc-ATP:

Preparation of Boc-NH(CH₂)₆N(H)COFc (Compound 1): Ferrocenecarboxylic acid (230 mg, 1 mmol) was dissolved in 20 mL anhydrous DCM. Then, 1.2 equiv. TEA (0.17 mL) and 1.2 equiv. HBTU (455 mg) were added sequentially. After 30 min., Boc-NH(CH₂)₆NH₂ was added to the solution and stirring was continued overnight. After reaction was completed, the solvent was removed *in vacuo*, and the residue was purified by flash column chromatography on silica gel (DCM-MeOH, 95:5; R_f = 0.25) giving the desired compound as a yellow solid in 78% yield (334 mg). ¹H-NMR (δ, DMSO): 7.74 (t, 1H, J = 5.2 Hz, NH-COFc), 6.78 (t, 1H, J = 5.4 Hz, NH-Boc), 4.78 (s, 2H, Cp), 4.32 (s, 2H, Cp), 4.14 (s, 5H, Cp), 3.15 (q, 2H, J = 6.4 Hz, CH₂), 2.90 (q, 2H, J = 6.4 Hz, CH₂), 1.23-1.52 (m, 17H). ¹³C{¹H}-NMR (δ, DMSO): 168.57, 155.57, 77.27, 76.94, 69.73, 69.23, 68.06, 39.76, 38.54, 29.50, 29.47, 28.26, 26.17, 26.08. IR: ν_{max} = 3363 (NH), 3310 (NH), 2976 (Fc), 2934 (Fc), 2861 (Fc), 1687 (CO-O^tBu), 1623 (Amide-1), 1535 (Amide-2). MS(EI+) *m/z*: calc. for C₂₂H₃₂FeN₂O₃: 428.2; found: (M+) 428.1

Preparation of NH₂(CH₂)₆N(H)COFc (Compound 2): TFA (5 equiv.) as added to a mixture of Boc-protected ferrocenyl amine (334 mg, 1 mmol) in 10 mL DCM. After stirring the mixture for 1 h, the solvent was removed *in vacuo*. Three portions of DCM were added and evaporated to get rid of the excess TFA. The residue was dissolved in 10 mL DCM and 0.25 mL TEA was added to convert the TFA salt to free amine completely. After solvent removal, the mixture (contains TEAH⁺ salt) was used in the next step without further purification. For the purpose of characterization, the mixture was dissolved in 20 mL DCM (contains 5% TEA) and extracted with brine and water. After the removal of the solvent, the residue was dried *in vacuo* to give a yellow solid. 90% yield (295 mg). ¹H-NMR (δ, DMSO-d₆): 7.74 (t, 1H, J = 5.3 Hz, NH-COFc), 4.78 (s, 2H, Cp), 4.32 (s, 2H, Cp), 4.14 (s, 5H, Cp), 3.15 (q, 2H, J = 6.4 Hz, CH₂), 2.52 (s, 2H, CH₂), 1.49 (t, 1H, J = 6.6 Hz, CH₂), 1.27-1.39 (m, 6H, CH₂). ¹³C{¹H}-NMR (δ, DMSO): 168.56, 76.95, 69.70, 69.21, 68.05, 41.53, 38.58, 33.23, 29.51, 26.41, 26.24. IR: ν_{max} = 3293.68(NH₂), 2962.94(Fc), 2928.63(Fc), 2854.23(Fc), 1624.45(amide-1), 1541.51(amide-2). MS(EI) *m/z*: calc. for C₁₇H₂₄FeN₂O: 328.1; found: (M+) 328.1.

Preparation of γ-phosphate Fc-ATP (Compound 3): Adenosine 5'-triphosphate disodium salt (100 mg, 0.18 mmol) was dissolved in 10 mL 0.1 M TEAB buffer (pH=7.5) and loaded on a column packed with cation-exchange resin (AG 50W-X8), which has been pre-equilibrated with 0.1M TEAB buffer. The desired fraction (monitored by UV light) was collected and evaporated *in vacuo*. The residue was co-evaporated with 10 mL dry methanol three times and dissolved in 1.8 mL dry DMF under Argon. DCC (123 mg) was added and the mixture was stirred under Ar for 3 h at room temperature to form adenosine-5'-trimetaphosphate (ATMP). ATMP solution was added to a mixture of compound 2 (295 mg, 5 equiv.) in 10 mL MeOH and 0.25 mL TEA under Ar. The mixture was stirred for 30 min. and poured into 20 mL H₂O. The solution was loaded on a DEAE-cellulose column and washed with distilled H₂O to remove excess ferrocenamine. Then, linear gradient of TEAB buffer (0.1-1 M) was carried out to give the desired

fraction (yellow band), which was lyophilized into light yellow powder. 50% yield of Fc-ATP (TEAH⁺ salt) and further exchanged TEAH to Na form for the NMR spectra. ³¹P{¹H}-NMR (δ, D₂O): -0.07(γ) d, J = 21.1 Hz; -10.76(α) d, J = 19.9 Hz; -22.14(β) t, J = 19.9 Hz. ¹H-NMR (δ, D₂O): 8.52 (s, 1H, H-8), 8.19 (s, 1H, H-2), 6.10 (d, 1H, J = 5.5 Hz, H-1'), 4.73 (s, 2H, Cp), 4.74 (s, 1H, H-2'), 4.53 (s, 1H, H-3'), 4.46 (s, 2H, Cp), 4.37 (s, 1H, H-4'), 4.23 (m, 2H, H-5'), 4.20 (s, 5H, Cp), 3.16 (t, 2H, J=6.5Hz, CH₂), 2.79 (q, 2H, J = 7.8 Hz, CH₂), 1.31-1.45 (m, 4H, CH₂), 1.11-1.25 (m, 4H, CH₂). [H₄M](ESI⁺) m/z: calc. for C₂₇H₃₉FeN₇O₁₃P₃: 818.1; found: 818.2.

Kinase-catalyzed phosphorylation reaction using Fc-ATP:

In order to avoid rapid evaporation of the solutions on the surfaces, the SPEs were incubated in petri-dishes at room temperature throughout the preparatory steps. The electrochemical measurements were performed three times for each condition (n=3), except otherwise stated. The results show the average of measurements with the error bars indicating the relative standard deviation (RSD).

Immobilization of the substrate peptides on SPEs

An aliquot of 200 μM substrate peptide solution (5 μL) was allowed to coat the gold working electrode of the SPEs and kept overnight at 4°C. After the incubation step, the electrodes were washed with blank TBS. The peptide film was diluted by immersing the SPEs in 0.1 mM ethanolic solution of hexanethiol for 5 min and rinsing the surface with blank TBS.

PKC-catalyzed phosphorylation on the SPE surface

Kinase assay buffer included 20 mM Tris, 0.5 mM EDTA, 10 mM MgCl₂, 500 μg/mL phosphatidyl serine (pH 7.5). The concentrations of Fc-ATP and PKC were varied according to the optimum experimental conditions. The aliquots (200 μL) of the optimized assay buffer including 100 U/mL PKC and 100 μM Fc-ATP were added into 1.5-mL vials. Substrate peptide-immobilized SPEs were placed in the vials incubated at 30°C for 1 h in a heating block (VWR Scientific, USA). After 1 h of incubation, the SPEs were washed with blank TBS to remove the excess Fc-ATP and other reagents, and then placed in the electrochemical workstation.

Electrochemical measurement on SPCE surface

Electrochemical detection was performed by spotting 20 μL of 0.1 M NaClO₄ (pH 6.5) onto the surface of SPE at room temperature. Cyclic voltammetry (CV) was performed at a scan rate of 100 mV/s. Square-wave voltammetry (SWV) involved the oxidation of Fc residues by sweeping the potential from 0 to 1 V with an amplitude of 25 mV at 15 Hz frequency.

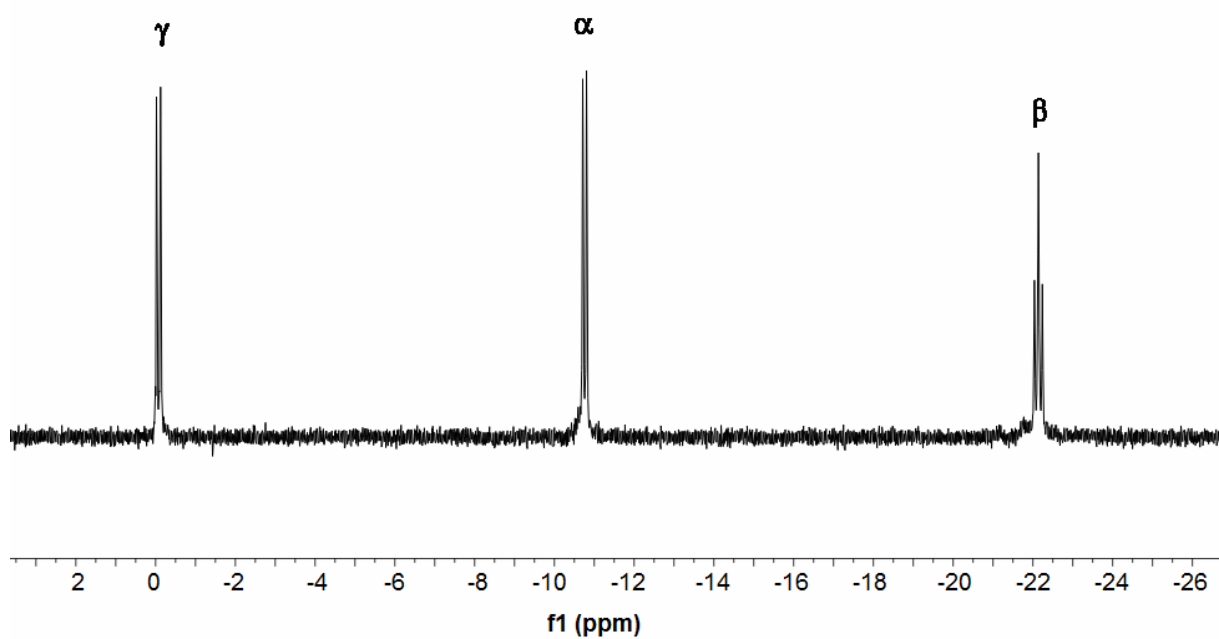


Fig. S1: $^{31}\text{P}\{^1\text{H}\}$ NMR of Fc-ATP sodium salt.

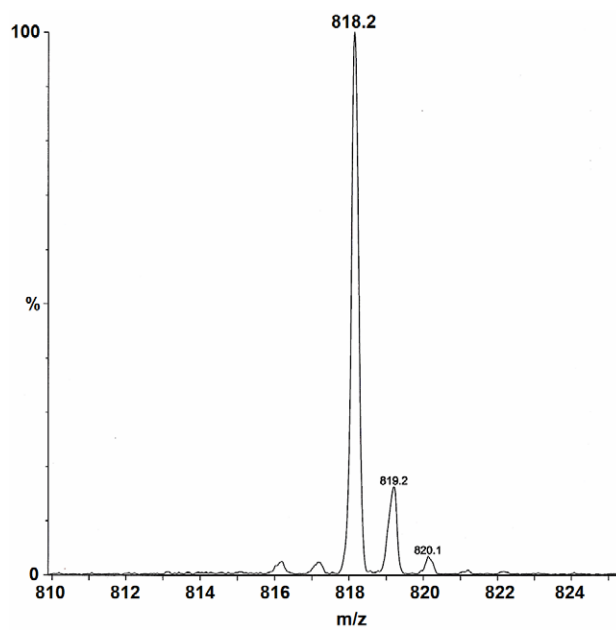


Fig. S2. Positive ion TOF-MS of Fc-ATP measured at 135 V.

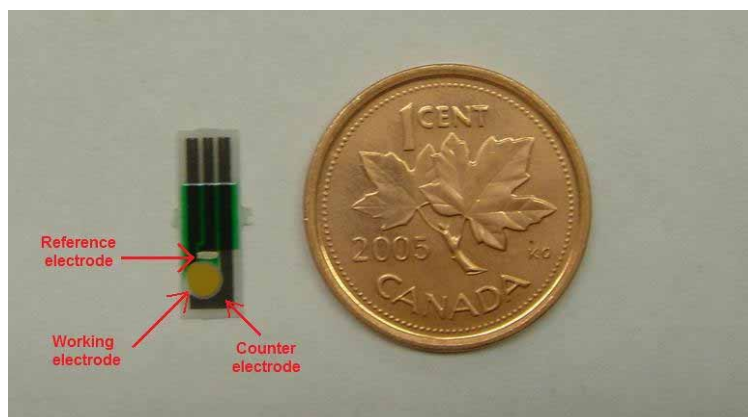


Fig. S3. DEP-Chip with a screen-printed gold electrode (SPE).

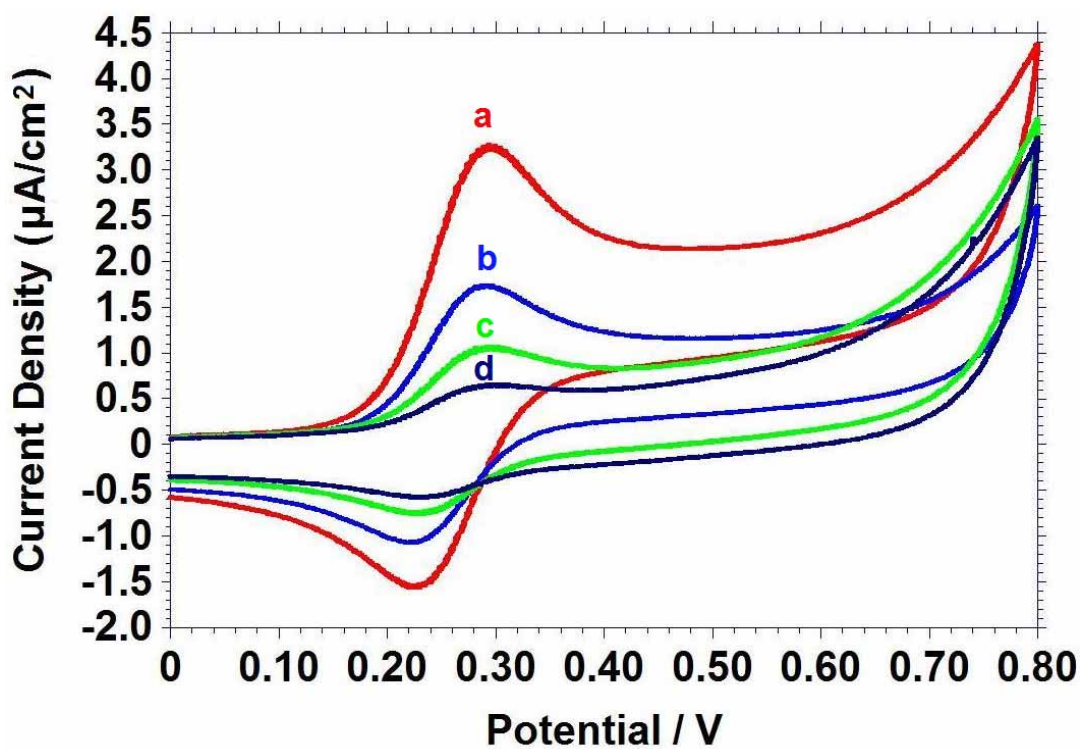


Fig. S4(A). Cyclic voltammograms of Fc-ATP in 0.1 M NaClO_4 . The CV measurements were taken at a scan rate of 100 mV/s using bare gold SPE in the presence of (a) 100 μM , (b) 50 μM , (c) 25 μM and (d) 10 μM Fc-ATP in solution.

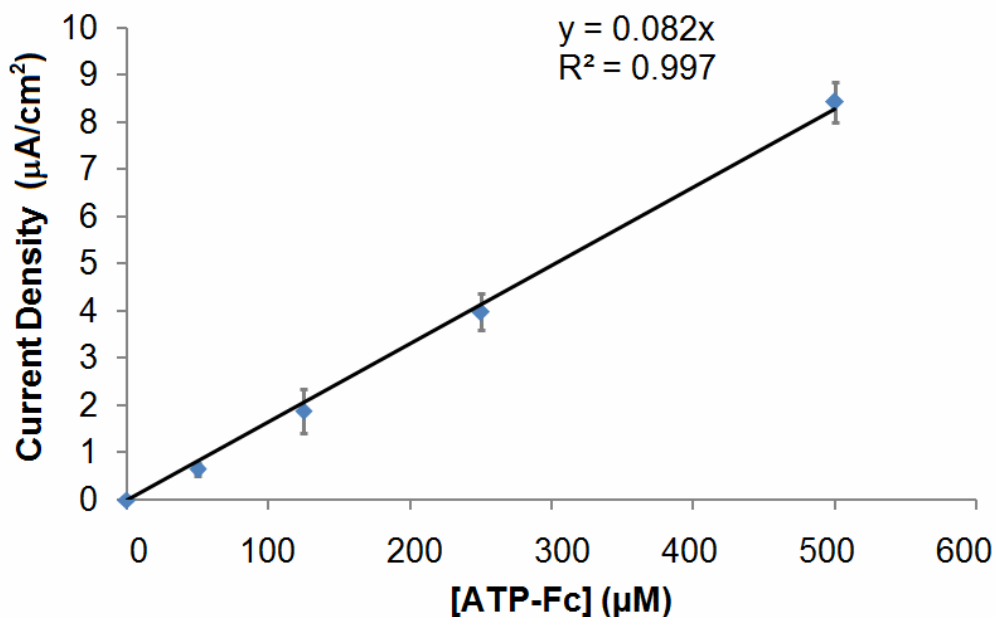


Fig. S4(B). Plot for the dependence of current density signals on the concentration of ferrocene-conjugated ATP in solution at a bare gold SPE. Other conditions were as described in ESI Fig. 4A.

The CVs exhibited quasi-reversible redox reactions as the anodic to cathodic peak current ratios were near unison. The peak width at half-maximum of Fc-ATP showed $101(\pm 9)$ mV, which was close to the ideal redox behaviour of 90 mV.

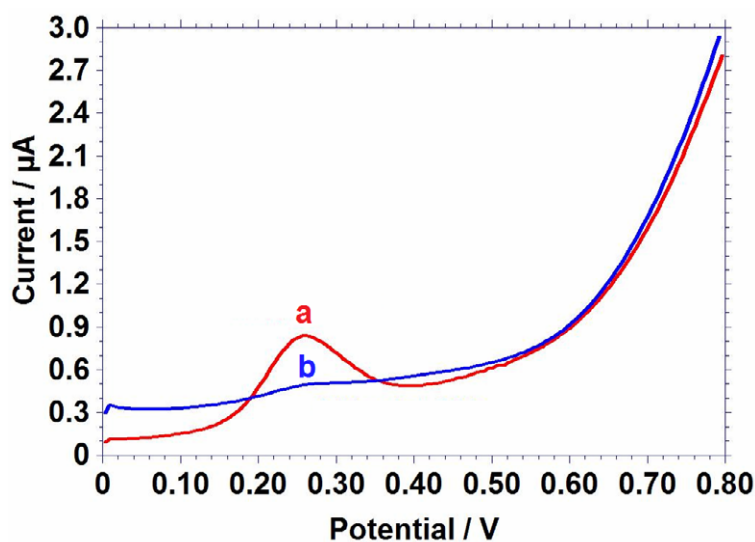


Fig. S5. Square-wave voltammograms of Fc-ATP on SPEs modified with 200 µM substrate peptide in the presence of PKC at (a) 0.1 U/mL, and (b) 0.01 U/mL. Other experimental

conditions were as described in the Experimental. SWV measurements were taken at a frequency of 15 Hz.

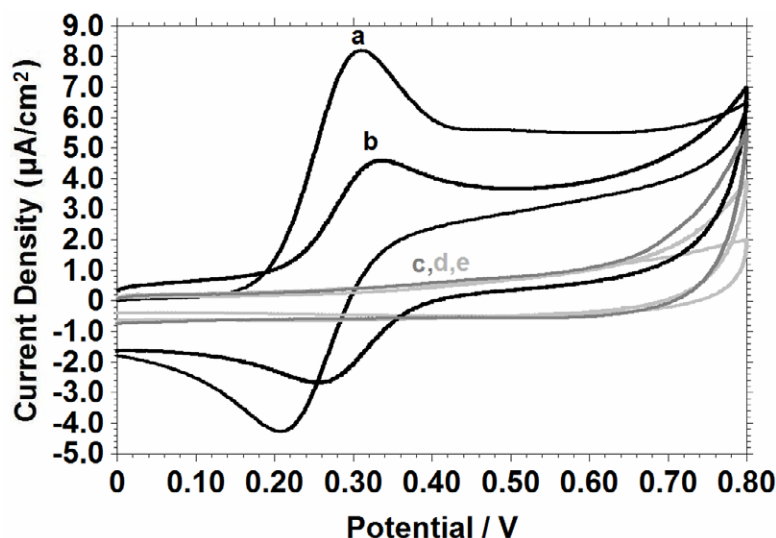


Fig. S6. Cyclic voltammograms for the PKC-catalyzed phosphorylation reactions in the presence of 100 U/mL PKC at varying concentrations of Fc-ATP, (a) 100 μM , (b) 50 μM , (c) 5 μM , (d) 1 μM and (e) no Fc-ATP. Other experimental conditions were as described in the Experimental.

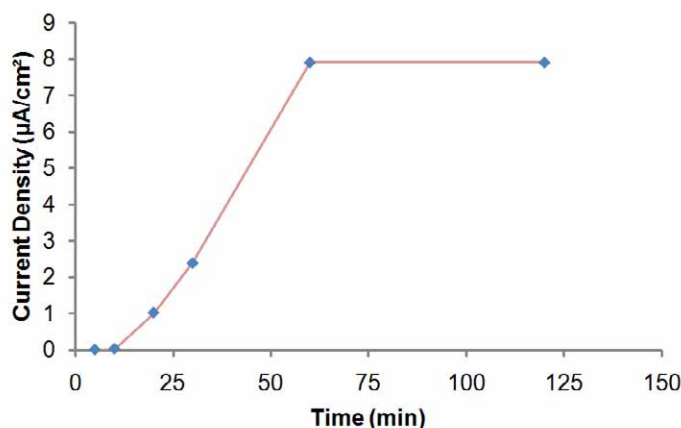


Fig. S7. Plot for the dependence of current density responses on the incubation times of the PKC-catalyzed phosphorylation reactions on SPEs at 30°C. Other conditions were as described in the Experimental.

References:

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Supplementary Material (ESI) for Chemical Communications
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