Following protein kinase acivity by electrochemical means and contact angle measurements†

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The electrochemical analysis of the protein kinase, casein kinase, is accomplished by the voltammetric response of Ag^+ ions associated with the phosphorylated product; the sensing surface is regenerated by the cleavage of the phosphorylated product with alkaline phosphatase, and the phosphorylation/de-phosphorylation processes are monitored by XPS and contact angle measurements.

The phosphorylation of proteins by protein kinases plays a major role in signal transduction and the activation of intracellular processes.[1] The over-expression of protein kinases was reported to cause various diseases such as cancer,[2] or Alzheimer's disease.[3] Thus, the sensitive and rapid detection of protein kinases attracts substantial research interest in bioanalysis. Different detection schemes for analyzing the activities of protein kinases were reported, and these included immuno-assays that involved phosphate-specific fluorophore-labeled antibodies that bind specifically to the phosphorylated proteins,[4] or a radio-isotope assay that used radioactive ATP for the phosphorylation reactions.[5] Recent reports described a fluorescent polarization assay to probe protein kinases,[6] an electrochemical method that used ferrocene-labeled ATP as redox tracer of the phosphorylation,[7] and the phosphorylation of the peptide with a thiophosphate that binds Au nanoparticles that are analyzed by voltammetry.[8] Also, an analytical protocol to follow the depletion of ATP upon the biocatalyzed phosphorylation of the protein,[9] and an assay of protein kinase that used gold nanoparticles[10] were reported. Similarly, the cyclic, label-free, detection of protein kinase was accomplished on a field-effect transistor device.[11] In the present study we report on the sensing of the casein kinase activity by the electrochemical reduction of silver ions linked to the phosphorylated sites of a protein monolayer associated with the electrode. By a second method, we demonstrate the use of contact angle measurements to follow the protein kinase activity. We also demonstrate the reusability of the sensing surfaces by the de-phosphorylation of the phosphate sites and the regeneration of the phosphate-free peptide through the application of alkaline phosphatase (ALP). We characterize the system by X-ray photoelectron spectroscopy (XPS) and quartz crystal microbalance (QCM) measurements.

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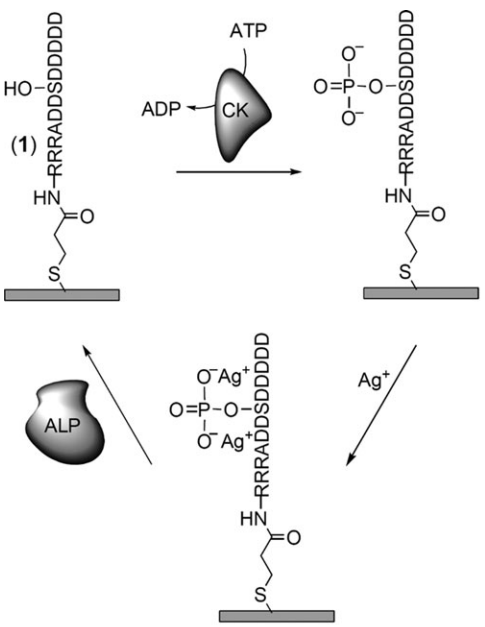
The electrochemical method to analyze the activity of casein kinase (CK) is depicted in Scheme 1. An Au electrode was functionalized with the peptide (**1**) by the modification of the electrode with the thiolated *N*-hydroxysuccinimide active ester monolayer, followed by the coupling of the peptide (**1**) to the surface. The peptide is recognized by CK. The electrode was treated with CK in the presence of adenosine triphosphate, ATP, to stimulate the phosphorylation of the serine residue. The resulting phosphorylated monolayer-modified electrode was electrochemically analyzed by the reduction of the bound Ag^+ ions. Quartz crystal microbalance measurements indicated that the surface coverage of the peptide on the Au electrode corresponded to 3×10^{-10} mol cm^{-2}.

The (**1**)-functionalized electrode was reacted with a constant concentration of CK and ATP for different time-intervals, and the time-dependent phosphorylation of the peptide was followed by the electrochemical reduction of Ag^+ ions linked to the phosphate residues. Fig. 1(A) depicts the square wave



(**1**) Arg-Arg-Arg-Ala-Asp-Asp-Ser-Asp-Asp-Asp-Asp-Asp

(**2**) Gly-Leu-Ala-Aib-Ser-Gly-Phe-Pro-Arg-Gly-Arg-Tyr

Scheme 1 Analysis of the phosphorylation of the peptide, (**1**)-monolayer electrode by CK/ATP and de-phosphorylation of the monolayer by alkaline phosphatase using Ag^+ as electrochemical label; (**2**) is a foreign peptide sequence, not recognized by CK.

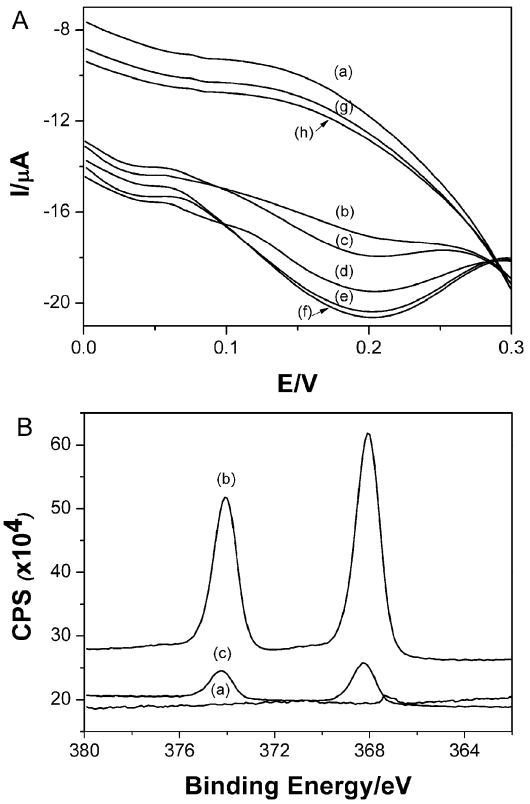


Fig. 1 (A) Square wave voltammograms (SWV) corresponding to the time-dependent phosphorylation of the (**1**)-functionalized Au electrode by CK, 20 units, and ATP, 1×10^{-4} M, at 30 °C for (a) 0 min; (b) 3 min; (c) 5 min; (d) 10 min; (e) 15 min and (f) 20 min. (g) Corresponds to the SWV of the Ag^+-phosphorylated monolayer electrode after treatment with alkaline phosphatase, 25 units for 30 min. (h) Corresponds to the SWV of the foreign peptide (**2**)-functionalized Au electrode after treatment with CK, 20 units, and ATP, 1×10^{-4} M, at 30 °C for 20 min, and the interaction of resulting electrode with Ag^+. In all experiments the phosphorylated monolayer electrodes were incubated in a $AgNO_3$ solution, 5 mM, for 30 min, and the resulting electrodes were analyzed in a HEPES buffer solution, 50 mM, as electrolyte using SCE as reference electrode. (B) XPS spectra corresponding to: (a) the (**1**)-functionalized Au-surface; (b) the phosphorylated monolayer surface generated by CK, 20 units, and ATP, 1×10^{-4} M, at 30 °C for 20 min after incubation with Ag^+ ions and (c) the surface generated by the treatment of the Ag^+-phosphorylated peptide monolayer with alkaline phosphatase, 25 units for 30 min.

voltammograms corresponding to the reduction of the Ag^+ ions linked to the phosphate units generated upon interaction of the modified surface with CK/ATP for variable time-intervals. As the reaction with CK/ATP is prolonged, the reduction wave is intensified, and the current values reach saturation after *ca.* 20 min of reaction, a time-interval that leads to the complete phosphorylation of the monolayer. Control experiments reveal that exclusion of either CK or Ag^+ from the system do not lead to the reduction wave at 0.2 V *vs.* SCE. These results imply that the reduction of Ag^+ occurred only upon the phosphorylation of the monolayer. The re-oxidation of the resulting Ag^0 nano-clusters resulted in the Ag^+-phosphorylated monolayer electrode, and the surface was treated with alkaline phosphatase.

Fig. 1(A), curve (g), depicts the voltammogram of the resulting electrode. Evidently, the Ag^+ ions were removed from the surface upon the enzyme-induced cleavage of the phosphate groups. As a result of the alkaline phosphatase-stimulated cleavage of the phosphate groups, the (**1**)-functionalized monolayer-modified surface was regenerated, and it was re-applied to analyze the phosphorylation by casein kinase with no significant loss of the electrode performance. The phosphorylation of the peptide (**1**) by CK/ATP is specific. In a further control experiment the electrode was modified with foreign peptide (**2**), and the (**2**)-modified electrode was reacted with CK/ATP, and subsequently treated with Ag^+ ions. The SWV of the resulting electrode is shown in Fig. 1(A), curve (h). The voltammogram is almost overlapping the voltammogram of the (**1**)-modified electrode that was not reacted with CK but interacted with Ag^+, Fig. 1(A), curve (a). These results indicate that phosphorylation of (**1**) by CK/ATP is specific. Interestingly, a minute voltammetric wave is apparent at 0.04 V and is observed at the CK-untreated (**1**)-functionalized electrode as well as the (**2**)-modified electrode. This wave is attributed to the reduction of Ag^+ ions associated to the amide bonds of the peptides. Also, treatment of the (**1**)-functionalized electrode with CK in the absence of ATP, yields after treatment with Ag^+ a minute response. All these control experiments imply that the voltammetric response originates from the association of Ag^+ to the phosphorylated sites.

The phosphorylation of the (**1**)-functionalized electrode by CK/ATP, and the association of Ag^+ to the monolayer, as well as the de-phosphorylation of the monolayer in the presence of alkaline phosphatase were monitored by X-ray photoelectron spectroscopy (XPS), Fig. 1(B). The association of Ag^+ to the phosphorylated monolayer yields the characteristic $Ag\,3d_{5/2}$ and $Ag\,3d_{3/2}$ bands at 368 and 374 eV, respectively, Fig. 1(B), curve (b). Treatment of the Ag^+-bound monolayer surface with alkaline phosphatase removed the phosphate groups, resulting in only residual Ag^+ signals, Fig. 1(B), curve (c).

The method to follow the CK activity by the electrochemical response of the associated Ag^+ ions was applied for the

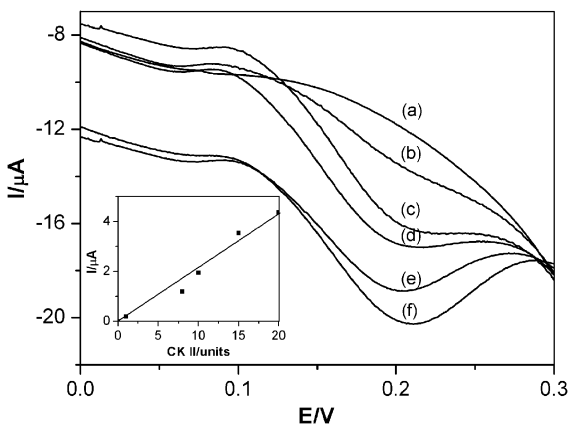


Fig. 2 SWV corresponding to the analysis of different concentrations of CK through the electrochemical reduction of Ag^+ ions linked to the phosphorylated product: (a) 0; (b) 1 unit; (c) 8 units; (d) 10 units; (e) 15 units; (f) 20 units. Inset: Derived calibration curve. The experimental conditions are detailed in the caption of Fig. 1(A).

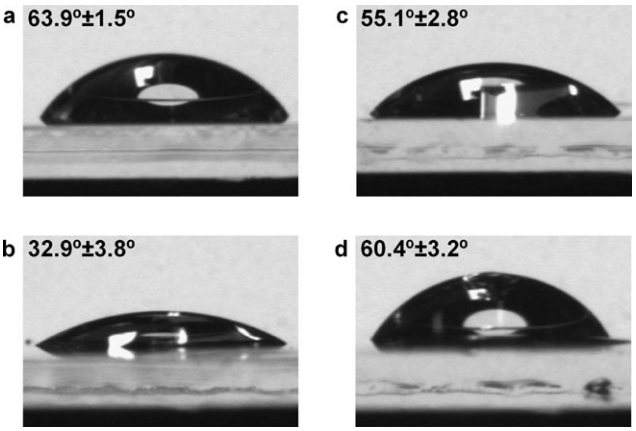


Fig. 3 Image of aqueous HEPES buffer droplets (20 μl) on the corresponding surfaces. (a) The (**1**)-functionalized monolayer electrode. (b) The phosphorylated (**1**)-peptide monolayer-modified surface. (c) The phosphorylated surface after treatment with Ag^+. (d) The Ag^+-phosphorylated monolayer modified surface after treatment with alkaline phosphatase. The experimental details are given in the caption of Fig. 1(A).

quantitative assay of CK, Fig. 2. The (**1**)-functionalized electrode was reacted for a fixed time-interval of 30 min with variable concentrations of CK in the presence of ATP, 0.1 mM. The resulting electrodes were treated with Ag^+, and the resulting voltammograms were used for the quantitative analysis of the phosphorylation process. As the concentrations of CK are higher, the resulting currents observed for the reduction of the Ag^+ ions in the SWVs are intensified. The resulting calibration curve is depicted in Fig. 2, inset. The method enabled the analysis of CK with a detection limit that corresponds to 1 U.

Finally, the analysis of CK activity, and the alkaline phosphatase-mediated de-phosphorylation process of the CK-generated product were also followed by contact angle measurements, Fig. 3. The water droplet positioned on the (**1**)-functionalized Au surface exhibited a contact angle that corresponded to 63.9 ± 1.5°, Fig. 3(a). After treatment of the surface with CK/ATP, the phosphorylated surface revealed a contact angle of 32.9 ± 3.8°, Fig. 3(b), consistent with the formation of a surface with enhanced hydrophilicity. The binding of Ag^+ to the phosphorylated monolayer-modified surface altered the contact angle to 55.1 ± 2.8°, Fig. 3(c). The decrease in the hydrophilicity of the surface is attributed to the fact that the tight Ag^+-phosphate bond masks the ionic nature of the phosphate groups, thereby turning the surface to a state of reduced hydrophilicity.[12] Treatment of the system with alkaline phosphatase removed the phosphate units and regenerated the original peptide-modified surface. This was reflected by the contact angle 60.4 ± 3.2°, Fig. 3(d), that indicated the formation of a surface exhibiting increased hydrophobicity.

In conclusion, the present study has developed an electrochemical assay for protein kinases that is based on the reduction of Ag^+ ions associated with the enzyme-generated phosphorylated sites. Our study has demonstrated the use of XPS and contact angle measurements as effective physical tools to characterize the phosphorylation process as well as the de-phosphorylation of the protein kinase-generated product by alkaline phosphatase.

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