Gold Nanoparticle Enhanced Electrochemical Assay for Protein Kinase Activity Using a Synthetic Chemosensor on a Microchip

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The phosphorylation of proteins regulates almost all aspects of cell life. Protein kinases are enzymes which catalyze the phosphorylation of target proteins. Mutations in particular protein kinases give rise to a number of disorders; and many naturally occurring toxins, and pathogens exert their effects by altering the phosphorylation states of intracellular proteins.1,2 Unusual regulation of protein phosphorylation is associated with many diseases, including cancer.3 Therefore, accurate quantification of the protein kinases is crucial for the analysis of related abnormalities.

Radioactive labeled adenosine triphosphate (γ32P-ATP) is the most commonly used strategy for detecting protein kinases, which involves probing of γ32PPO3 incorporated in the phosphorylated peptide. Traditionally, gel-based assays, filter-binding assays, or enzyme-linked immunosorbent assays have been used for such purposes.4,5 These processes typically require half to a full day, and highly trained personnel to perform these specialized multiple analytical steps. In addition, these consume a lot of expensive reagents and require a number of specialized and often expensive instruments. Therefore, there is a critical need to miniaturize and automate such systems.

Recently, kinase activity assays based on mass spectrometry,6 fluorescence,7 and quartz crystal microbalance8 have been reported for the analysis. However, miniaturization of these techniques for handheld device is quite not possible. μTAS technologies have been widely explored to develop miniaturized devices for portable use. Recent advances in microfabrication technique have facilitated the creation of microchips with electrochemical detection of different biomarkers.9,10 Electrochemical detection enable rapid, cost-effective, and high throughput screening.11–13 To detect protein kinases electrochemically, a variety of methods have been proposed. The most common approach for such analysis is the use of ferrocene tagged adenosine triphosphate (ATP).14,15 On the similar code, Sun et al. used a biotin tagged ATP while Kerman and Kraatz used a thiol tagged ATP.16,17 Recently, Xu et al. and Nam et al. also reported DNA and aptamer based protein kinase detection.18,19 Though all these reported techniques proved to be effective, they require labor-intensive label- ing procedures or preparation of the electrode surface. Most of these protocols use biomolecules as a recognizing and reporting molecule which reduces the commercial value of the protocols. Another major drawback in the field of kinase biosensor is the lack of lab-on-a-chip devices. As of now, only a few groups developed a microchip based protein kinase sensor.20,21 For this reason, this work focuses on the development of a microchip for protein kinase assay with a minimalistic use of biomolecules.

To specifically detect protein kinase, a couple of groups have reported the use of synthetic chemosensor.22,23 In the past, we also described a simple and versatile strategy for monitoring kinase activity using phosphate-selective fluorescent quenchers.24 Recently, we reported a modified chemosensor for the electrochemical detection of protein kinase A.25 This chemosensor facilitated the electrochemical detection, instead of an optical based quantification (Fig. 1b). Briefly, the chemosensor has a bis(Zn2+–dipicolylamine) group with an affinity for the phosphate group at one end and a thiol group at the other self-assembling onto the surface of the gold electrode. The two Zn2+ cations selectively binds to the phosphate group by forming a bridge.

The present work reports a further improved microchip with enhanced sensitivity for the electrochemical assay of protein kinase using the same chemosensor. The sensitivity of the quantification was enhanced by using gold nanoparticles (AuNPs). AuNPs have attracted considerable interest for research in bioanalytical applications due to their excellent biocompatibility.26,27 NPs are known to increase active surface area thereby increasing the probe binding site. They also aid in signal transfer to the electrode and enhancement.28,29 Thus, chemosensor was anchored with its thiol end on the gold working electrode through gold nanoparticle bridge. A self-assembled monolayer of nanoparticles can be easily formed using any dithiol linker and still retain the biochemical activity of the labeled molecules.

Therefore, in this work, we demonstrate a homogeneous assay for protein kinase A using the synthetic chemosensor immobilized on the AuNPs modified gold working electrode. Most of the previous reports were based on heterogeneous catalysis, in which the enzyme phosphorylates the peptide substrate fixed on the solid surface. Our method here, employs the homogeneous enzymatic catalysis where the enzyme and the substrate react together in free form, and then the chemosensor immobilized on the electrode selectively captures the product. cAMP–dependent protein kinase A (PKA) was used as a model enzyme in our study. A commercially known PKA-specific peptide, fenrofenylated kemptide (Fc-CO-LRRASLG-NH2), was used both as the phosphorylation substrate and the reporter for the PKA activity. Upon oxidation, the phosphorylated-fenrofenylated peptide produced an electrochemical signal. The voltammetric study confirmed that the higher the concentration of the enzyme, the more phosphorylated product bound to the chemosensor. Thus, a highly sensitive and selective platform for PKA was obtained in this study.

Experimental

Materials and equipment.— cAMP–dependent protein kinase A and protein kinase buffer was purchased from New England Biolabs.
Results and Discussion

The proposed electrochemical kinase assay using chemosensor is presented in Fig. 2. For the deposition of AuNPs, the electrodes were first rinsed thoroughly with isopropanol, DI water and dried under nitrogen (N₂). Au electrodes were initially dipped in 10 mM ethanolic solution of 1,6-hexanediol for 12 hrs in dark at room temperature to form a thiol terminated monolayer. After thoroughly rinsing with ethanol for several minutes, the electrodes were dried under N₂ and then dipped into the AuNPs colloidal solution for 12 hrs. The AuNPs-1,6-HDT-gold electrodes were rinsed with DI water and dried under N₂. Each modification step of electrode surface was confirmed using cyclic voltammetry (CV). CV after each modification step was performed in 5 mM K₄[Fe(CN)₆] prepared in 10 mM potassium chloride at a scan rate of 100 mV s⁻¹ (Fig. S2). The three layers of microchip were then aligned as discussed before. After finalizing the fabrication, the chemosensor was injected into the electrode cell and was then incubated for the required amount of time (Fig. S3) at 5 °C. The modified electrodes were rinsed with the buffer and used for kinase assay.

PKA detection using microchip.— The reaction mixture in the presence of 1X PKA buffer was pumped into the reaction chamber and incubated at 30 °C for 1 hour to phosphorylate the substrate. To achieve the desired temperature for incubation, a calibrated DC power supply was applied to the ITO layer (Table S1). The phosphorylated product was then introduced to the electrochemical cell containing chemosensor modified electrode, for 30 min. Finally the DPV study was carried out in carbonate buffer (0.1 M, 9.2 pH) to measure the change in electrochemical signal due to enzymatic catalysis. The DPV study was performed in a scan range of 0–0.5 V, with an increment of 0.002 V, amplitude of 0.05 V, and pulse width of 0.04 s. As expected, the mixture produced a well-defined current signal peak.

Functionalization of electrode.— The device schematic, structure of synthetic chemosensor, and proposed electrochemical kinase assay using chemosensor is presented in Fig. 1 and Fig. 2. The UV-visible spectra, SEM and TEM image of the AuNPs is shown in Fig S1. According to the analysis, AuNPs showed a diameter of approximately 13 nm with narrow size distribution. To confirm the electrode modification steps and the properties of the resulting chemosensor, CV in 5 mM K₄[Fe(CN)₆] after each step was performed (Fig. S2). CV of K₄[Fe(CN)₆] using the gold electrode after each modification gave different electrochemical responses. After the deposition of ethanolic 1,6-HDT on the bare gold electrode, current signal from K₄[Fe(CN)₆] reduced greatly due to the binding of molecules to the electrode surface and less availability of free gold. On the contrary, self-assembly of AuNPs on 1,6-HDT restored the current signal and was higher than that of the bare gold electrode. This proves that the AuNPs provide a higher surface area and enhances the electron transfer to the electrode surface.
Figure 2. Schematic strategy of electrode modification and electrochemical assay of protein kinase using chemosensor on microchip, Fc: ferrocene tagged kemptide and P: phosphate group.

Optimization of experimental parameter.— To determine the deposition time of chemosensor on AuNPs, the electrodes were incubated with chemosensor for different amount of time. After formation of the layers, the chemosensor modified electrodes were rinsed with the buffer and allowed to react with positive kemptide for 30 min (chemically phosphorylated peptide, Fc-CO-LRRApSLG-NH₂). Finally, differential pulse voltammetry (DPV) was performed to measure the extent of deposition. The change in peak current was monitored to establish the immobilization time. As shown in Fig S3, the peak current went up with the increase in deposition time up to 6 hrs. However, the peak current saturated when the deposition time was beyond 6 hrs. Thus, 6 hrs were selected as the optimal for depositing chemosensor on gold electrode for further experiments.

ATP concentration is an important parameter during the kinase-catalyzed reaction, as it donates the phosphate group for the phosphorylation. To optimize the concentration of ATP, a series of reactions were performed with different concentrations of ATP in the presence of 50 U mL⁻¹ of PKA and kemptide. The PKA phosphorylated the kemptide using ATP. The analyses were performed on AuNPs/chemosensor modified electrode. As can be seen in Fig. 3, the peak current went up with the increase in ATP concentration up to 250 μM, with a saturation beyond that. Therefore, 250 μM was adjudged as the optimal ATP concentration.

The reaction time of the PKA catalyzed phosphorylation determines the performance of the electroanalysis. The optimization of reaction time was studied in the range of 0–75 min in the presence of 50 U mL⁻¹ of PKA. As shown in Fig. 4, the peak current went up with the increasing reaction time, reaching a saturation at 60 min. The saturation of signal symbolizes the complete phosphorylation of the kemptide. Therefore, a reaction time of 60 min was used in the further work. Since the ATP concentration and reaction time depends on the concentration of substrate kemptide, its amount was kept same in all the analyses.

Protein kinase A activity assay in microchip.— For the enzymatic assay, DPV study was carried out to measure a change in the electrochemical signal by enzymatic catalysis. Under the optimized experimental parameters, the reaction mixture (1X PKA buffer, 250 μM ATP, 40 μM kemptide, PKA enzyme) with different concentrations of PKA was prepared in the reaction chamber and allowed to react at 30 C. In order to validate the signal enhancement in PKA detection owing to nanoparticles, Fig. 5 compares the signal obtained with AuNPs monolayer and without AuNPs.25 It was found that the sensitivity and signal output significantly increased due to the use of AuNPs. Fig. 6a, illustrates the dependence of voltammetric current obtained from AuNPs modified electrodes on the concentrations of

Figure 3. DPV results of optimization of ATP concentration; Reaction condition: 1X PKA buffer, 40 μM kemptide, 50 U mL⁻¹ PKA.

Figure 4. DPV results of optimization of reaction time; Reaction condition: 1X PKA buffer, 40 μM kemptide, 50 U mL⁻¹ PKA.
PKA. The mixture produced a well-defined increasing peak current at 0.25 V corresponding to the electrochemical oxidation of the ferrocenyl group proportional to the increase in the PKA concentration (0.05 U mL\(^{-1}\) to 50 U mL\(^{-1}\)). Higher signal represents the increased amount of phosphorylation in the presence of increasing concentration of PKA. The higher is the phosphorylation, the more kemptide will bind to the chemosensor functionalized electrode. Fig. 6b exhibits the enzyme activity plotted using the data collected from Fig. 6a. An observable detection limit of PKA was estimated to be 0.05 U mL\(^{-1}\) (S/N = 3, n = 3) with a linear range from 2 U mL\(^{-1}\) to 50 U mL\(^{-1}\) (R\(^2\) = 0.98; Fig. 6b inset). A number of control experiments confirmed that this current peak originated only when the ferrocenylated positive or the PKA catalyzed kemptide existed in the solution. No significant signal was obtained from the product incubated in the absence of PKA. This result signifies that the signal change is undisturbed by the nonspecific adsorption of the peptide substrate on the electrode surface.

**Selectivity and sensitivity of microchip.**—In order to further demonstrate the potential practicality, the experiments were performed in the presence of human blood. Freshly drawn human blood was mixed with the reaction mixture containing different concentrations of PKA and incubated in the microchip as described earlier. The enzymatic reaction product was then analyzed voltammetrically using the AuNPs and chemosensor modified electrode. Table I summarizes the analyses, demonstrating good recoveries with respect to the concentrations of spiked PKA. A marginally elevated signal was obtained which is likely due to the presence of inherent kinase enzyme in the blood. Nonetheless, the device showed high signal to noise ratio with chemosensor precisely distinguishing the phosphorylated kemptide and other interfering biomolecules present in the blood. To further study the potential application of this microchip, an inhibition study was performed in the presence of 10 mM EDTA mixed in the reaction mixture. EDTA in the medium acts as a chelator of divalent cations (Mg\(^{2+}\)), which inhibits the activity of kinase and thereby preventing the phosphorylation. Addition of EDTA significantly reduced the voltammetric signal, signifying the halting of phosphorylation (Data not given). This proved the successful miniaturization of PKA assay using synthetic chemosensor on a polymeric microchip. The proposed microchip can be applied for the determination of PKA for routine clinical diagnosis and can easily be modified to assay any other protein kinase with their specific protein substrate.

### Conclusions

In conclusion, this work describes a strategy for developing a polymeric microchip for electrochemical assay for protein kinase activity using AuNPs and synthetic chemosensor functionalized electrode. The enzyme catalyzed phosphorylated ferrocene-kemptide upon binding to chemosensor produces a voltammetric signal, which can quantify the PKA. The developed method offers attractive features like homogenous assay, phosphate selective chemosensor and costless polymeric microchip. A polymer based microchip and a synthetic probe microchip can be applied for the determination of PKA for routine clinical diagnosis and can easily be modified to assay any other protein kinase with their specific protein substrate.

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### Table I. DPV of PKA activity on ferrocenylated kemptide in the presence of blood.

<table>
<thead>
<tr>
<th>Enzyme added (U mL(^{-1}))</th>
<th>Current expected (A)</th>
<th>Current obtained (A)</th>
<th>Signal recovery (%)</th>
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<tbody>
<tr>
<td>1</td>
<td>(1.33 \times 10^{-7})</td>
<td>(1.45 \times 10^{-7})</td>
<td>109</td>
</tr>
<tr>
<td>5</td>
<td>(2.29 \times 10^{-7})</td>
<td>(2.66 \times 10^{-7})</td>
<td>116</td>
</tr>
<tr>
<td>10</td>
<td>(2.85 \times 10^{-7})</td>
<td>(3.14 \times 10^{-7})</td>
<td>110</td>
</tr>
</tbody>
</table>

[Figure 5. Plots of electrochemical signal values for 40 U mL\(^{-1}\) PKA analysis on bare electrode and AuNPs modified electrode.]

[Figure 6. (a) DPV of PKA activity on ferrocenylated kemptide with PKA on the gold electrode modified with the AuNPs and chemosensor. Reaction condition: 1X PKA buffer, 40 μM kemptide, 250 μM ATP; (b) Plot for the current response at 50, 40, 30, 20, 10, 05, 02, 01, 0.5, 0.2, 0.1, 0.05 and 0 U mL\(^{-1}\) of protein kinase A; The inset shows the linear range of PKA detection.]
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References