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Short Review

Probing of Protein Kinase Activity by Electrochemistry

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Protein kinase can catalyze the transfer of a phosphoryl group from adenosine triphosphate (ATP) to protein/peptide substrate at the serine, tyrosine or threonine residues. The phosphorylation can induce the surface charge change of substrate peptides-modified electrode; the reaction could be measured by the change of the electrochemical impedance or the tyrosine oxidation current. The phosphate groups can be selectively recognized by anti-phosphorylated peptide antibody and metal ions, metal complexes as well as metal nanoparticles through the coordination or electrostatic interaction, which facilitated the design of various electrochemical kinase biosensors. Furthermore, the γ-phosphate-modified ATP analogues (e.g. biotinylated, sulfated and ferrocene-labeled ATP) have been used as the co-substrates of phosphorylation to monitor protein kinase activity. All of these electrochemical strategies for kinase assays were summarized in the present work.

Keywords: kinase; phosphorylation; electrochemistry; adenosine triphosphate; self-assembled monolayers

1. INTRODUCTION

Protein kinase can catalyze the transfer of a phosphoryl group from adenosine triphosphate (ATP) to protein/peptide substrate at the serine, tyrosine or threonine residues. This reaction can lead to a functional change of the target protein by regulating enzyme activity, cellular location, or association with other proteins [1,2]. Thus, protein kinases play crucial roles in many biological processes, including signal transduction, cell apoptosis, immune regulation, proliferation and differentiation and other important cellular pathways [3,4]. The changes in the activities and contents of kinases have been associated with many diseases, such as cancer, metabolic disorders and

inflammation [5,6]. In recent years, electrochemical techniques have been exploited for the detection of protein kinase activity because of their high sensitivity, simplicity, rapid response, and compatibility with miniaturization. In this paper, we summarized various electrochemical methods for probing of kinase activity.

2. DIRECT DETECTION OF PHOSPHORYLATION

2.1 Phosphorylation-induced charge change of electrode surface

Phosphorylation catalyzed by protein kinase on self-assembly monolayers (SAMs)-covered electrode surface can induce the change of surface charge and electrochemical impedance. This will facilitate or block the penetration of the electrochemical probes ($Ru(NH_3)_5Cl]^{2+}$ or $[Fe(CN)_6]^{3-/4-}$) on the electrode surface [7]. For this view, several groups have presented the label-free electrochemical methods for the detection of protein kinase activity and screening of the inhibitors [8-12]. For example, Wang et al. found that the positively charged peptides formed a compact SAM on gold electrode surface, thus blocking the penetration of the positively charged $[Ru(NH_3)_5Cl]^{2+}$ [8]. When the peptides were phosphorylated catalytically by kinase, the positively charged SAMs became loose because of the presence of two negatively charged phosphate groups. As a result, $[Ru(NH_3)_5Cl]^{2+}$ could easily penetrate the SAM, thus producing a good electrochemical signal. Li et al. have demonstrated that the kinetics of phosphorylation and dephosphorylation mediated by kinases and phosphatases on electrode surface could be monitored by measuring the impedance change [10]. Moreover, Wilner et al. suggested that the phosphorylation process can be further amplified using the anti-phosphorylated peptide antibody to the monolayer (Fig. 1) [13]. In this work, the specific interaction between phosphorylated peptide and its antibody was also characterized by chemical force microscopy and contact-angle measurements. Then, the antigen-antibody interaction was used to determine phosphorylation in connection with enzyme-linked electrocatalysis using gold nanoparticles (AuNPs) as the carriers of streptavidin-labeled horseradish peroxidase (HRP) [14]. Also, it was used for the development of a microarray-based resonance light scattering (RLS) assay for determining kinase activity and inhibition [15].

It has been suggested that peptide with a special sequence can be digested by carboxypeptidase Y (CPY), but its phosphorylation by kinase could prevent the CPY-catalyzed digestion. Based on this principle, Yin et al. presented an electrochemical method for measuring casein kinase II (CK2) activity [16]. In brief, without phosphorylation, the peptide immobilized on electrode surface was digested by CPY. As a result, a strong electrochemical signal in $[Fe(CN)_6]^{3-/4-}$ was obtained. However, CK2-catalyzed phosphorylation stopped the digestion of peptide. Consequently, the peptide SAM on electrode blocked the penetration of $[Fe(CN)_6]^{3-/4-}$ probe and thus led to a low electrochemical current.

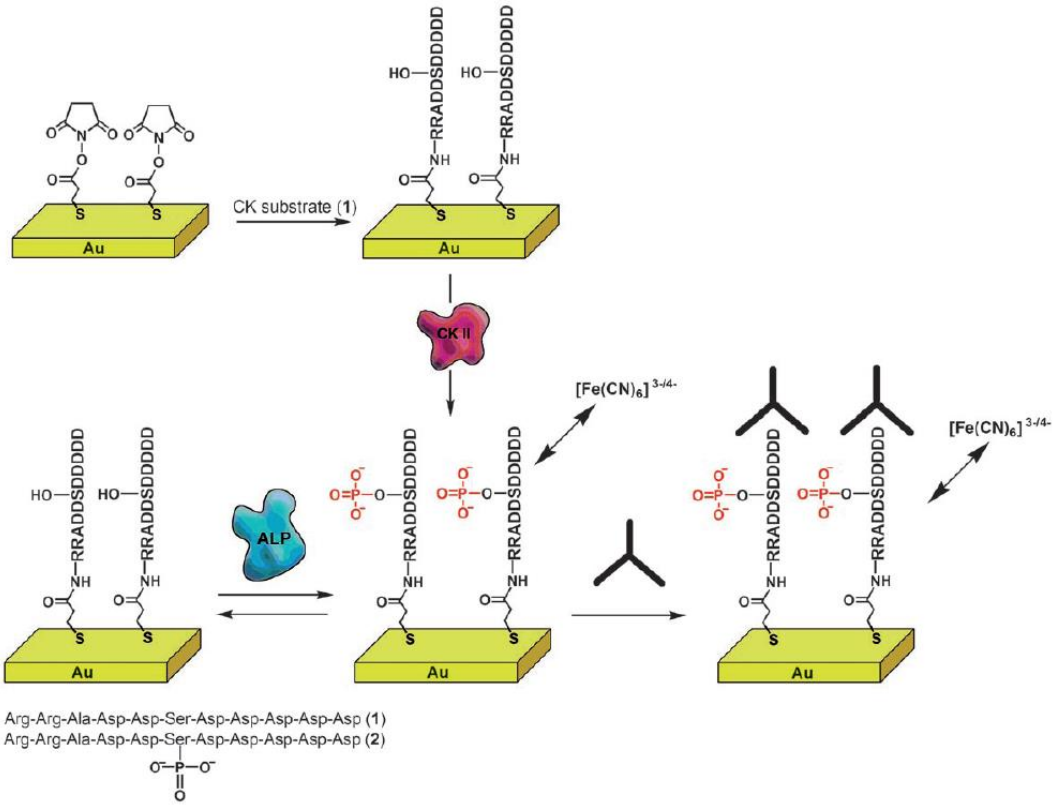

Figure 1. Analysis and amplification of casein kinase activity by electrochemical impedance spectroscopy (ALP=alkaline phosphatase). Reprinted with permission from [13]. Copyright 2008 John Wiley and Sons.

2.2 Tyrosine oxidation

Based on the fact that the tyrosine residue in a protein could be electrochemically oxidized [17,18], Kerman and co-workers investigated the electrochemical response of phosphorylated and non-phosphorylated peptides [19] and reported a method for the detection of tyrosine kinase activity [20]. As shown in Fig. 2, if the peptide substrate was phosphorylated at the position of tyrosine residue, the phosphorylated tyrosine would not be oxidized at a potential of 0.65 V. However, when the kinase activity was inhibited by a potential inhibitor, tyrosine will not be phosphorylated. As a result, a DPV current from the oxidation of tyrosine at ~0.65 V was observed on a multiwalled carbon nanotube-modified screen-printed carbon electrode (SPCE). This method wan then extended to determine the activity of cellular-sarcoma tyrosine non-receptor, a protein tyrosine kinase, and the inhibition efficiency of a well-known inhibitor, 4-amino-5-(4-chlorophenyl)-7- (*tert*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2). With the same detection principle, Li et al. determined a tyrosine kinase and its inhibitor using grapheme-modified glassy carbon electrodes [21]. The detection limit of 0.087 nM is lower than that obtained at the above multiwalled carbon nanotube-modified SPCE (5 U/mL). These results indicated that tyrosine residue is a good electrochemical signal reporter fro measuring the activity of protein tyrosine kinase. Furthermore, Qu et al. suggested that the electrochemical oxidation

of tyrosine could be catalyzed by employing $Os(bpy)_3^{2+}$ (bpy = 2,2-bipyridine) as the electron mediator [22]. The work should be helpful for the detection of kinases with improving sensitivity.

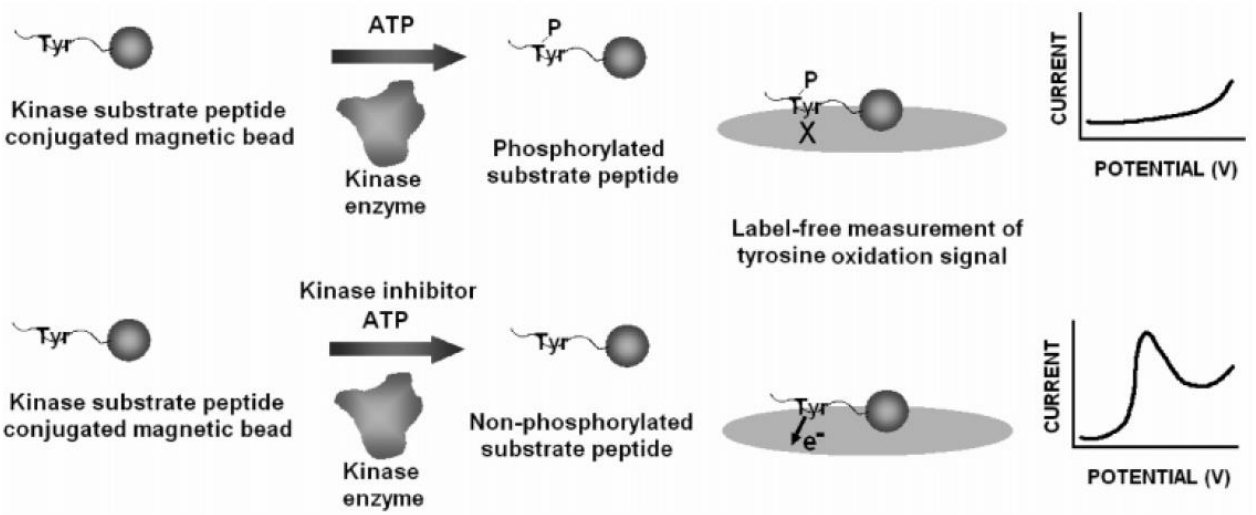

Figure 2. Schematic representation of the principle for the tyrosine (Tyr) oxidation-based detection of protein phosphorylation. Reprinted with permission from [20]. Copyright 2007 American Chemical Society.

Although many reports have demonstrated the oxidation of tyrosine, little research was found to investigate the electrochemical behaviours of phosphorylated tyrosine (Tyr-P) and sulfated tyrosine (Tyr-S) probably because of their high oxidation potential. Boron-doped diamond (BDD) electrode shows low background current, a wide working potential window, favorable electron transfer kinetics, and surface inertness [23]. Chiku et al. suggested that tyrosine, Tyr-P and Tyr-S show well-defined oxidation peaks at 0.8 V, 1.4 V and 1.7 V at the boron-doped BDD electrode, respectively [24]. Furthermore, they tested the activity of protein tyrosine kinase in human epidermoid carcinoma cells (A431) combined with peptide-modified magnetic beads. Without phosphorylation, the linear-sweep voltammogram shows only one oxidation peak from the tyrosine oxidation. After phosphorylation of peptide-modified magnetic beads by protein tyrosine kinase, a well-defined oxidation peak was observed at 1.4 V, while the peak current for tyrosine oxidation decreased significantly. Additionally, Popa and Diculescu studied the electrochemical behaviour of Tyr-P at a glassy carbon electrode over a wide pH range [25]. They found the oxidation of Tyr-P is an irreversible, pH-independent process involving the transfer of one electron and no proton. This work can provide important information for design of kinase sensors.

Moreover, tyrosine can be oxidized into L-DOPA (L-3,4-dihydroxyphenylalanine) and L-DOPA-quinone with tyrosinase as the oxidase, while Tyr-P is unrecognizable by tyrosinase. For this view, Wang et al. reported a simple and rapid electrochemical method for the determination of tyrosine kinase [26]. As shown in Fig. 3, tyrosinase catalyzed the production of the L-DOPA-quinone which could be reduced to L-DOPA at a potential of −0.2 V. The phosphorylation blocked the tyrosinase-catalyzed oxidation of tyrosine residue and thus inhibited the production of L-DOPA-quinone. With

the method, p60c-src protein tyrosine kinase was quickly measured in the concentration of 1.9−237.6 U/mL with a low detection limit of 0.23 U/mL.

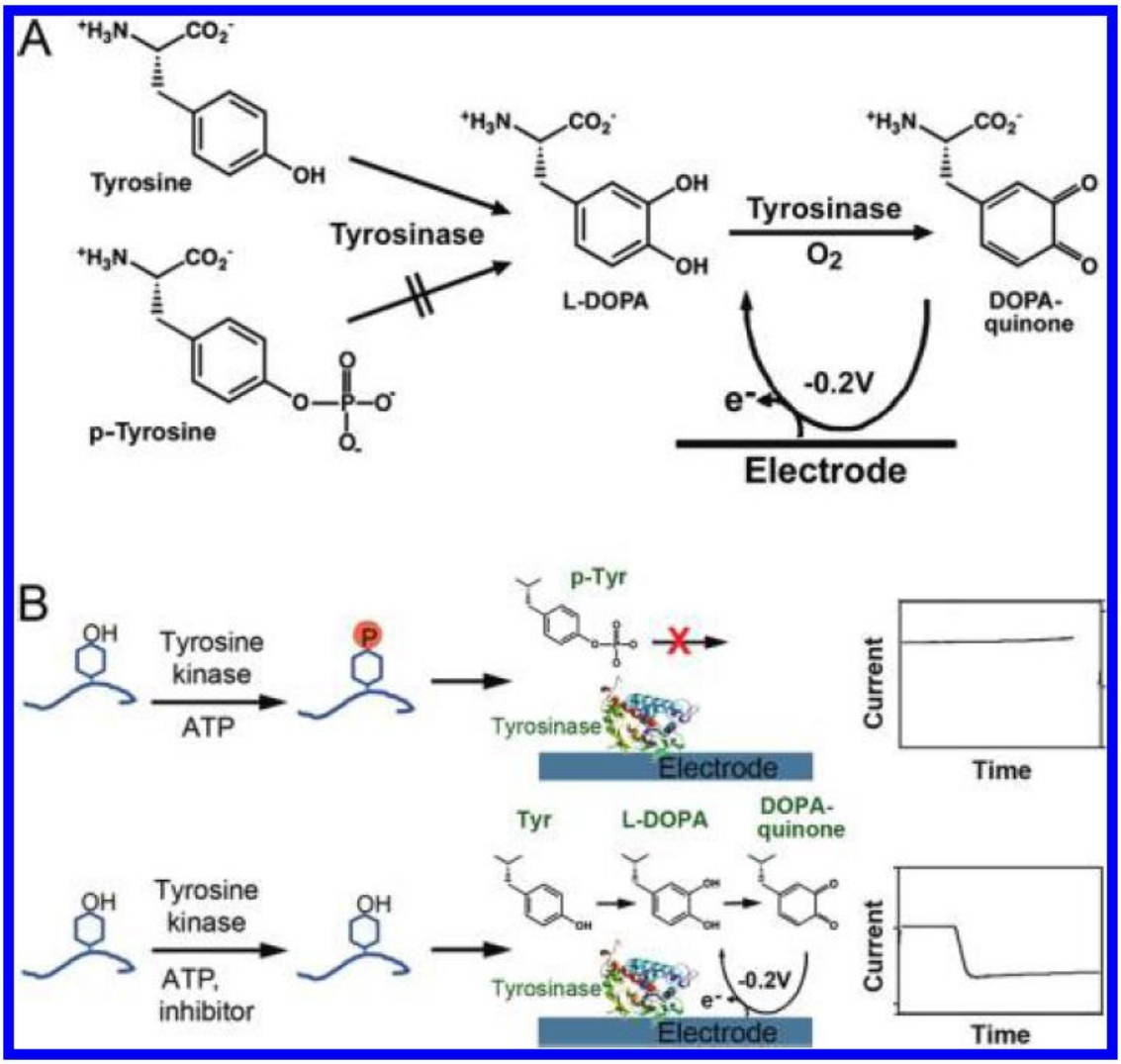

Figure 3. (A) Working model for the determination of the activity of protein tyrosine kinases and (B) schematic illustration of the principal for tryosinase-based detection of the phosphorylation of peptide. Reprinted with permission from [26]. Copyright 2012 American Chemical Society.

3. RECOGNITION OF PHOSPHATE GROUPS BY METAL IONS

Besides anti-phosphorylated peptide antibody, the phosphate group can also be recognized by metal ions, metal complexes and metal nanoparticles through the coordination or electrostatic interaction. The design of such chemosensors was summarized herein.

The bis(Zn^{2+}-dipicolylamine) complex is a good synthetic receptor that binds to phosphate group with high selectivity and strong affinity [27]. Modification of electrode with bis(Zn^{2+}-dipicolylamine) allowed for the capture of phosphorylated product. For example, Shin et al. presented

the "signal-on" assay of protein kinase activity by the thin film electrode modified with the bis(Zn^{2+}-dipicolylamine) complexes [28]. In this method, the peptide was labeled with a redox ferrocene tag. As shown in Fig. 4, only the phosphorylated peptide could be attached onto the electrode surface through the interaction of bis(Zn^{2+}-dipicolylamine) and phosphate group. Thus, protein kinase activity could be monitored by measuring the oxidation current of ferrocene. It has also indicated that AuNPs could be used as the bridge between the electrode and bis(Zn^{2+}-dipicolylamine) to facilitate the oxidation of ferrocene and enhance the electrochemical signal [29]. Conversely, bis(Zn^{2+}-dipicolylamine) complex can be used to recognize the phosphorylated peptide anchored on the electrode surface. Based on the bis(Zn^{2+}-dipicolylamine)-phosphate interaction, Yin et al. have demonstrated the detection of protein kinase activity with the biotin-labeled bis(Zn^{2+}-dipicolylamine) complex as the receptor. Through the strong biotin-avidin interaction between, avidin-conjugated horseradish peroxidase (HRP) was attached onto the electrode surface. This allowed for the catalytic oxidation of benzoquinone and produced a strong electrochemical signal [30]. Also, Yin et al. presented a photoelectrochemical biosensor for the determination of protein kinase activity by immobilizing the substrate peptide on the ITO electrode modified with visible-light active graphite-like carbon nitride (g-C3N4) and AuNPs [31].

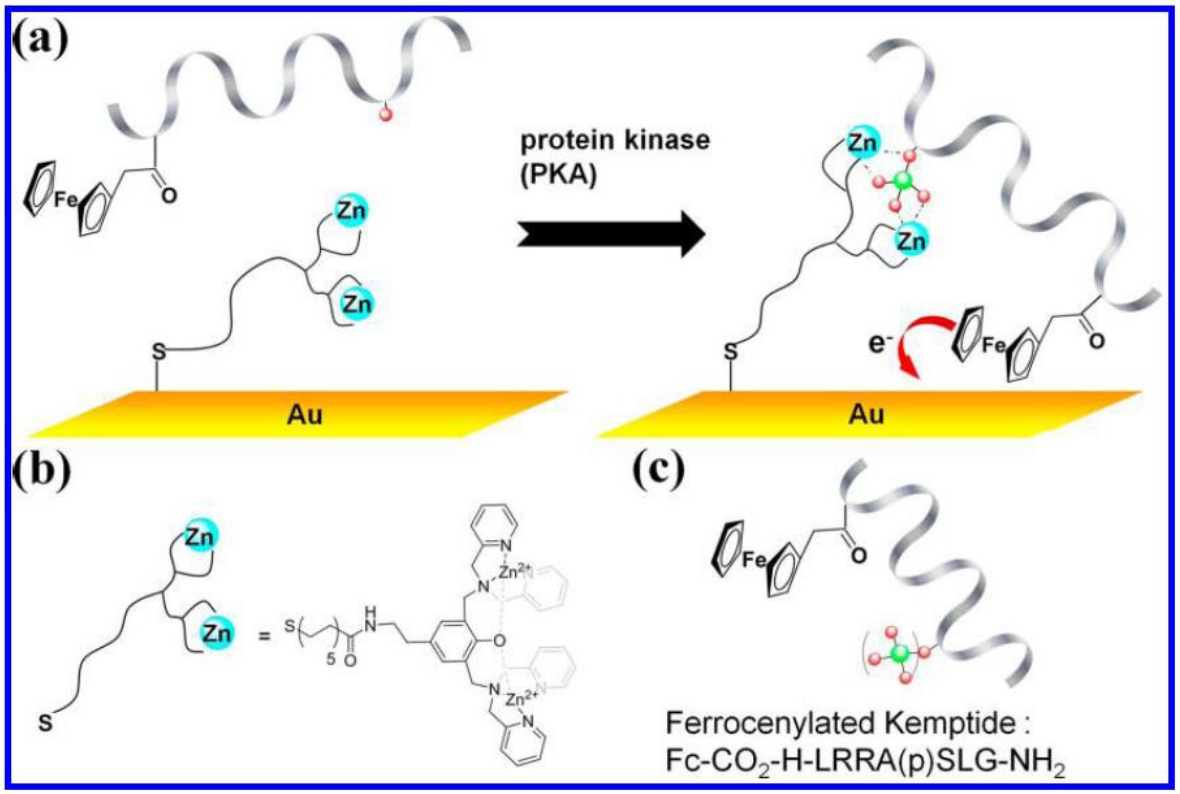

Figure 4. (a) Schematic strategy of homogeneous electrochemical assay for protein kinase activity, (b) structure of the phosphorylated peptidebinding receptor, and (c) ferrocenylated kemptide was used as a peptide substrate for PKA. Reprinted with permission from [28]. Copyright 2014 American Chemical Society.

Zirconium cation (Zr^{4+}) can be used as a capture regent for the enrichment of phosphorylated peptide [32]. Xu et al. designed the first Zr^{4+}-based electrochemical sensor for probing of kinase activity [33]. In this method, Zr^{4+} acted as a phosphorylation indentifier and a bridge of phosphate groups in the phosphorylated peptide immobilized on electrode and DNA assembled on AuNPs. The DNA–AuNPs were used as the reporters and enhancers for the loading of a large number of $[Ru(NH_3)_6]^{3+}$ through the electrostatical interaction. The electrochemical signal of $[Ru(NH_3)_6]^{3+}$ was collected by chronocoulometry. Based on the Zr^{4+}-mediated linkage between phosphorylated peptide and DNA-AuNPs, Wang et al. also presented a new signal-amplified strategy by the formation of a polymeric network of AuNPs on electrode surface [34]. In this design, the DNA1-AuNPs were first coupled with the phosphorylated peptide by Zr^{4+}. Then, the polymeric network of AuNPs was formed by the hybridization of cDNA with DNA1 and DNA2 on the nanoparticles surface. The amperometric response of $[Ru(NH_3)_6]^{3+}$ in the network is dependent upon the kinase activity. Additionally, Miao et al. proposed an electrochemical strategy for probing of protein kinase activity by Zr^{4+}-mediated signal transition and rolling circle amplification (RCA) [35]. As shown in Fig. 5, after the peptides attached on the electrode surface were phosphorylated by kinase, DNA primer probes were then captured by the electrode with Zr^{4+} as the linkage of the phosphorylated peptides and the DNA probes. In the presence of the padlock probes and phi29 DNA polymerases, RCA occurred on the electrode surface. This resulted in the formation of long DNA strands on electrode surface. The negatively charged long DNA strands would absorb large amounts of positively charged $[Ru(NH_3)_6]^{3+}$ through electrostatic interaction, which thus amplified the electrochemical signal.

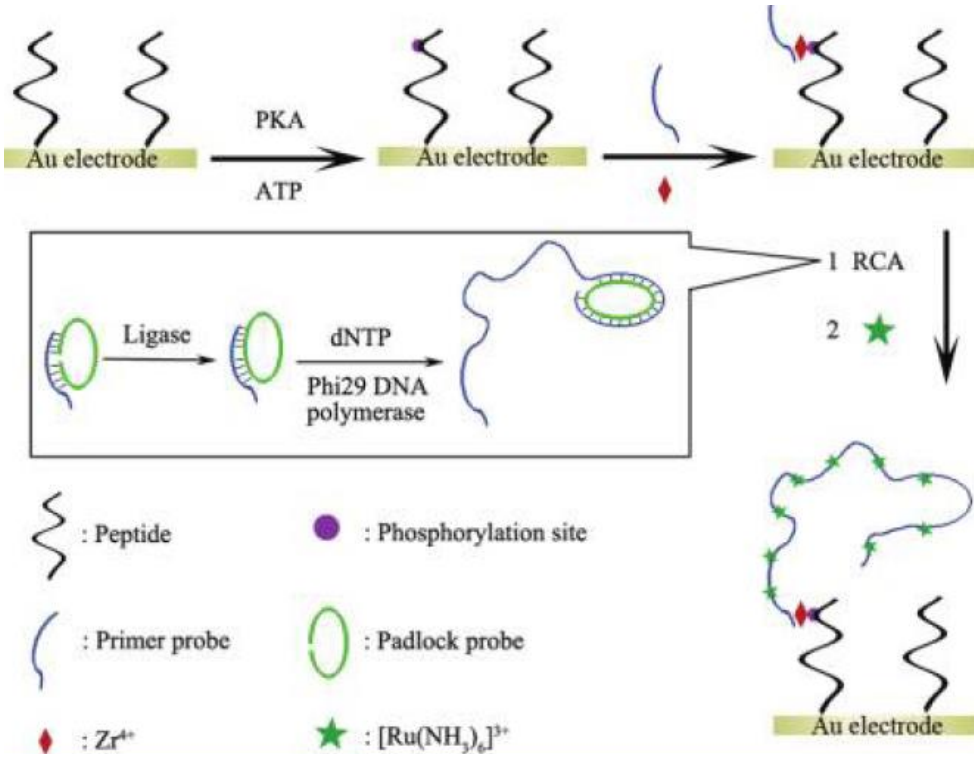

Figure 5. Schematic illustration of the strategy for monitoring the activity of protein kinase. Reprinted with permission from [35]. Copyright 2012 American Chemical Society.

Moreover, based on Zr^{4+}-triggered assembly of phosphorylated peptides and nanomaterials, such as graphene quantum dots [36], mesoporous SiO_2 microspheres [37], gold nanoclusters [38], DNA-AuNPs [39, 40] and Ru(II) encapsulated phosphorylate-terminated silica nanoparticles (R-PSiNPs) [41], other biosensors (e.g. photoluminescence, chemiluminescence, fluorescence and electrochemiluminescence) have also been made to monitor the activity of protein kinases. Typically, Yan et al. reported a visible-light photoelectrochemical (PEC) biosensor for the assay of protein kinase activity (Fig. 6) [40]. In this work, DNA-AuNPs were assembled on the phosphorylated peptides-covered TiO_2/ITO electrode surface through the chelation interaction between Zr^{4+} ions and phosphate groups. The $[Ru(bpy)_3]^{2+}$ was then intercalated into the DNA groove to produce excited electron. Because of the excellent conductivity and large surface area of AuNPs, the electron-transfer and loading amount of $[Ru(bpy)_3]^{2+}$ were improved. Thus, the photocurrent was significantly amplified.

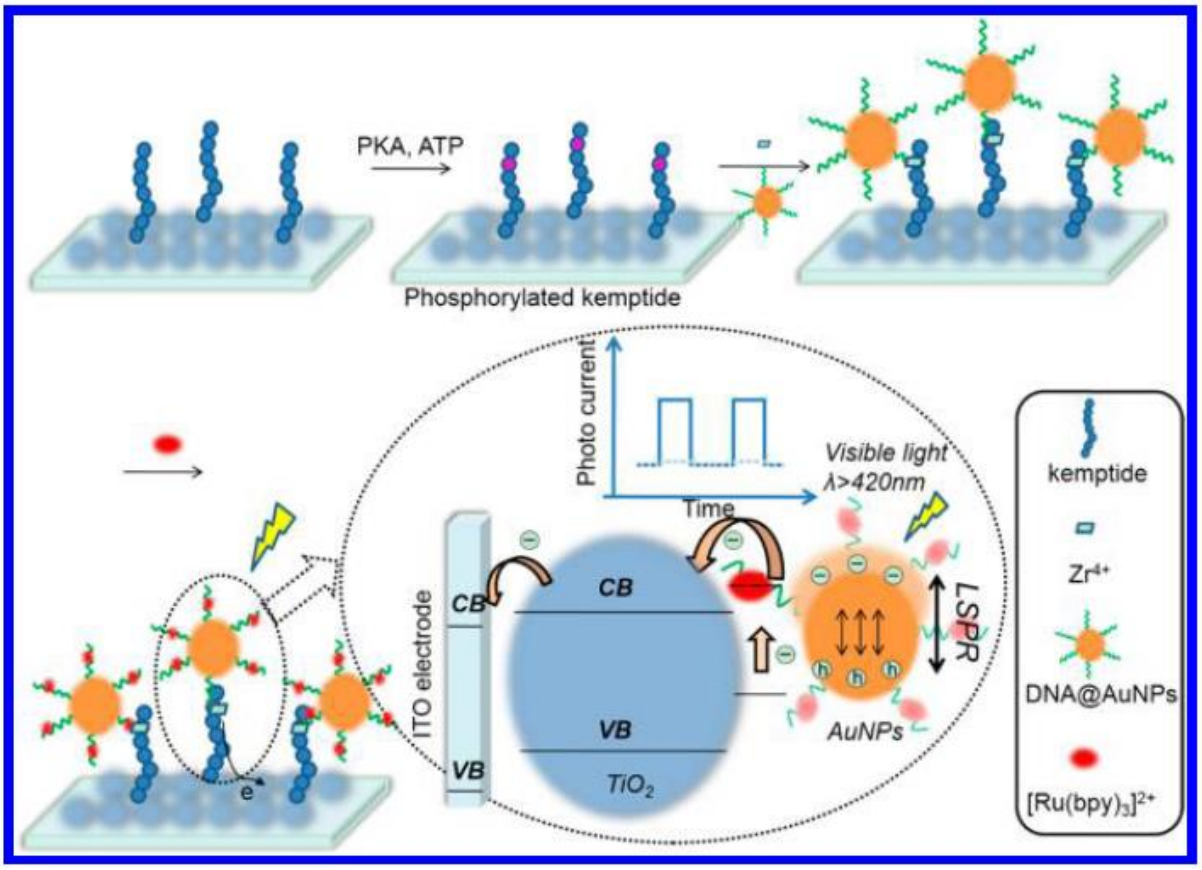

Figure 6. Configuration of photoelectrochemical biosensor for kinase activity detection. Reprinted with permission from [40]. Copyright 2016 American Chemical Society.

Besides Zr^{4+} ion, Fe^{3+}, Ag^{+} and Tb^{3+} ions also showed high affinity to the phosphorylated peptides. Thus, based on the interaction of metal and phosphate group, various novel electrochemical kinase biosensors could be fabricated. Typically, Wang et al. suggested that Fe^{3+} could bind to the phosphorylated peptides immobilized on electrode surface to show high electrocatalytic activity for the reduction of H_2O_2 [42]. Wieckowska et al. demonstrated that the phosphorylated monolayer-modified electrode can absorb Ag^{+}, thus facilitating the electrochemical reduction of Ag^{+} on electrode surface

[43]. Furthermore, TiO_2 has been used to the selective isolation of phosphorylated peptides based on its strong binding to phosphate group [44]. Ji et al. found that the phosphorylated peptides formed on electrode surface can be recognized by TiO_2. Then, Ag nanoparticles could be deposited on the TiO_2 surface and oxidized into Ag^+ during the electrochemical scanning.

The above metal ions have been traditionally used as phospho-affinity materials, but also showed nonspecific binding of nonphosphorylated peptides to some extent, especially the peptides containing acidic residues[45, 46]. Several groups have suggested that rare earth ions as the hard Lewis acids may serve as the candidates for the selective recognition of phosphorylated peptides [47-49]. For example, Zondlo et al. suggested that the binding affinity of Tb^{3+} to the phosphorylated peptide is 35-fold higher than to the nonphosphorylated peptide [47]. Zhang et al. studied the binding affinity of various rare earth ions to kinase-produced phosphopeptides by fluorescence imaging and found that Dy^{3+} showed the highest affinity [48]. Thus, Tb^{3+} and Dy^{3+} ions also show great potential in the design of new kinase biosensors.

4. ATP ANALOGUES AS THE CO-SUBSTRATED OF PHOSPHORYLATION

In phosphorylation process, a phosphoryl group from ATP is transferred to the specific amino residue of protein. Radio-labeled ATP is the currently used co-substrate for measuring protein kinase activity. Recently, other γ-phosphate-modified ATP analogues have been synthesized and used for probing of protein kinase activity, including biotinylated ATP (biotin-ATP), sulfated ATP (ATP-S) and ferrocene-labeled ATP (Fc-ATP). For example, phosphorylation of target protein/peptide with biotin-ATP allowed for the assays of kinases with streptavidin-conjugated reagents [50-52]. Using biotin-ATP (adenosine 5'-triphosphate [γ]-biotinyl-3,6,9-trioxaundecanediamine), Kerman et al. investigated the phosphorylation reaction based on the interaction between biotin and streptavidin-coated AuNPs. The attached AuNPs showed a good stripping peak in the presence chloride ion, enabling the monitoring of the kinase-catalyzed phosphorylation [53]. If ATP-S (adenosine 5'-[γ-thio] triphosphate) was used as the the co-substrate, the resulting thiophosphorylated peptide can capture unmodified AuNPs via the Au–S interaction. The AuNPs-induced electrochemical response also made it possible to measure the kinase activity [54-57]. Furthermore, the Au-S interaction was used to design an electrogenerated chemiluminescence (ECL) biosensor [58]. As shown in Fig. 7, AuNPs captured by the thiophosphate groups on electrode surface amplified the ECL signal of luminal oxidation because of their good conductivity, large surface area and excellent electroactivity. This work offered a sensitive ECL biosensor for kinase determination. In addition, the AuNPs/MWNTs nanohybrids as the reporters of thiophosphorylated peptide have also been fabricated and used for kinase assay with improving sensitivity [59]. The nanohybrids could be captured by the thiophosphorylated peptides and catalyzed the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) by H_2O_2, thus resulting in a great increase in the electrochemical current.

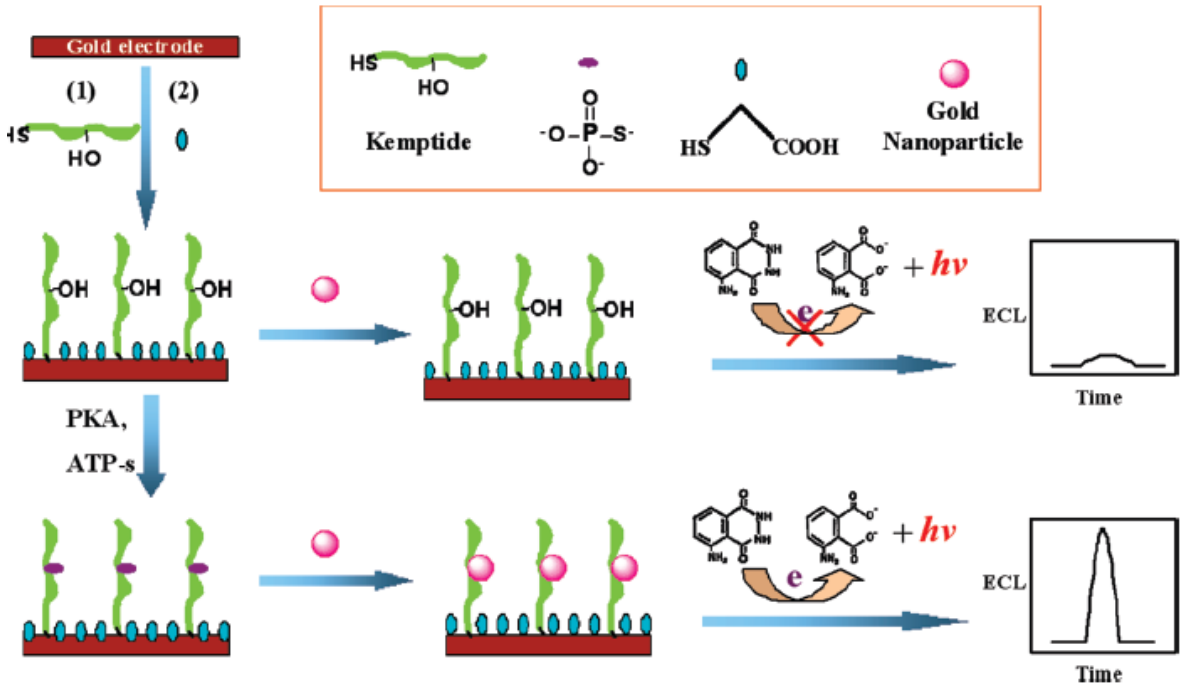

Figure 7. Schematic representation of ECL strategy for kinase activity detection using gold nanoparticles as signal transduction probes. Reprinted with permission from [58]. Copyright 2010 American Chemical Society.

Fc can be electrochemically oxidized at a low potential [60]. With Fc-ATP as the co-substrate, phosphorylation could be directly monitored by measuring the oxidation current of Fc. Kraatz's group first reported the synthesis of adenosine-5'-[γ-ferrocene] triphosphate in 2008 and used it as a co-substrate for detection of protein kinase by immobilizing a peptide substrate on electrode surface [61, 62]. With phosphorylation, the electroactive γ-phosphate-Fc group was transferred to the hydroxyamino acid in the side of peptide. The electrochemical response from the Fc oxidation enabled the determination of the activity of kinases (e.g. protein kinase C, casein kinase 2, protein kinase A, protein tyrosine kinases Abl1-T315I and HER2, glycogen synthase kinase (GSK-3b), and sarcoma-related protein kinases) and the screening of their potential inhibitors [61-66]. They also investigated the effect of the spacers between Fc and phosphoryl group of ATP on the phosphorylation reaction and found that a longer γ-Fc alkyl chain (C_6) alleviated the steric hindrance for its approaching to kinase [67, 68]. However, the hydrophilic poly(ethylene glycol) spacer is bad for the phosphorylation probably due to the additional H-bonding and electrostatic interaction between the poly(ethylene glycol) spacer and the kinase catalytic site and the substrate protein/peptide [68]. Furthermore, Kraatz and co-workers suggested that Fc could be recognized by a robust polyclonal rabbit antiferrocene antibody (Fc-Ab1) (Fig. 8) [69-71]. This work represents a novel idea for detection of protein kinase activity and inhibition by not only electrochemistry but also various optical techniques.

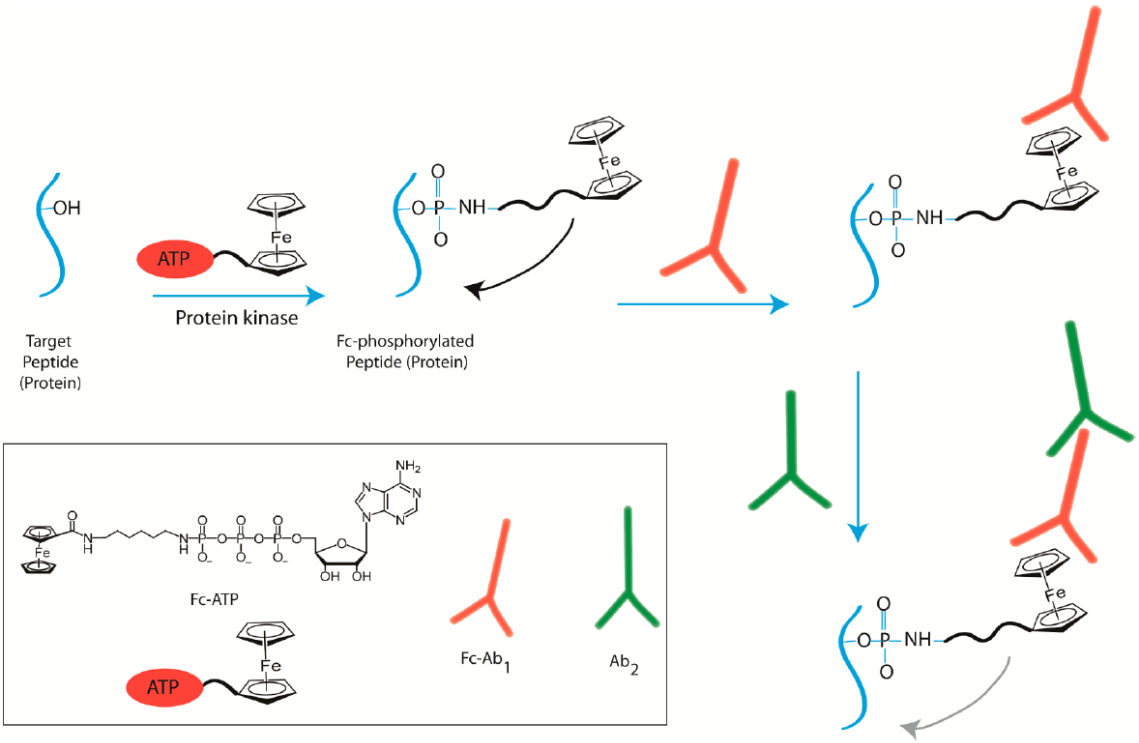

Figure 8. Strategy for Fc-phosphorylation of peptides and proteins. Target peptide or protein is Fc-phosphorylated in the presence of protein kinase and Fc-ATP cosubstrate. This process is successfully measured for the immobilized target by surface electrochemistry. In an immunoassay approach, primary polyclonal anit-ferrocene antibodies (Fc-Ab1) bind to Fc-phosphates, on surfaces or in solution, specifically followed by fluorescently labeled goat antirabbit antibodies (Ab2) binding in a two-step immunoreaction. On surfaces, the fluorescence imaging of Fc-phosphorylations, in an immunoarrray method, takes advantage of the proposed strategy. Similarly, the immunoreaction principle was utilized in a biochemical approach for Western blotting. Reprinted with permission from [69]. Copyright 2012 American Chemical Society.

5. CONCLUSION

We summarized the electrochemical strategies for detection of kinase activity. The phosphorylation could be monotored by measuring the kinase-induced change of tyrosine oxidation current or the surface charge of peptide immobizlied on electrode surface. The resulting phosphorylated peptides could also be recognized by the elements, such as ananti-phosphorylated peptide antibody, metal ions, metal complexes and metal nanoparticles. The use of ATP analogues as the co-substrates facilitated the developement of various novel electrocehmical methods. For assays of kinases in different samples and screening of novel inhibitors, more reliable information would be gleaned from the high-throughput assays, such as multichannel electrochemical cells with micro-fabricated and nanofabricated working electrodes.

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