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Mini review

Electrochemical Assay Methods for Protein Kinase Activity

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Protein phosphorylation catalyzed by protein kinase is the most frequent post-translational modification, and plays a critical role in intracellular signal transduction pathways. Deregulation of kinase activity is a frequent cause of various human diseases, including cancers. Moreover, kinase is widely regarded as a family of extremely important molecular target for drug therapy. Therefore, sensitive and specific detection of protein kinase activity and inhibition is essential to the prerequisite and foundation for the study of fundamental biochemical processes, the clinical diagnosis and drug discovery. In recent years, tremendous advances have been achieved in electrochemical biosensors for protein kinase activity. This article reviews the recent developments in the designed strategies for electrochemical detection of kinase.

Keywords: Phosphorylation; kinase; electrochemistry; biosensors

1. INTRODUCTION

Post-translational modification (PTM) can reveal the diversity and activity of proteins, clarify the way and mechanism of protein's function, and produce powerful biomarkers and targets for early diagnosis and treatment of some diseases [1, 2]. PTM can be divided into esterification, methylation, ubiquitination, glycosylation, acetylation and phosphorylation. These modification processes are carried out under the catalysis of kinases. Phosphorylation is the most common and important format of PTM in both prokaryotes and eukaryotes. The reaction occurs in all organisms, from fungi to

humans. In eukaryotic cells, about one third of proteins are modified by phosphorylation at the same time. There are about 100,000 potential phosphorylation sites in the human proteome.

Protein phosphorylation is catalyzed by protein kinase, which transfers the phosphoric acid group of ATP or GTP to the amino acid residue of substrate protein. Phosphorylation regulates almost all cell life processes, such as signal transduction, DNA replication, gene transcription regulation, protein synthesis, and cell cycle regulation [3]. Abnormal expression of kinase will lead to abnormal phosphorylation. It has been suggested that many diseases are caused by the abnormal phosphorylation modification of proteins or imbalance of phosphorylated protein expression, such as Alzheimer's disease, cancers and so on [4-6]. In contrast, the abnormal phosphorylation modification may be the result of some diseases. Therefore, the study of phosphorylation modification of proteins is of great significance for the diagnosis and treatment of many diseases.

2. ELECTROCHEMICAL STRATEGIES FOR KINASE DETECTION

Traditional methods for kinase activity assay include isotope ^{32}P labeling, mass spectrometry, autoradiography and gel electrophoresis [7]. These methods have been widely used in biological and medical experiments because of their accurate quantitative positioning. However, due to the shortcomings of expensive instruments, possible harm to human body, time-consuming and complex operation, the applications of these methods are limited. Thus, some new methods for kinase detection have been developed. As a classical biological analysis method, electrochemical biosensor has many advantages, such as good sensitivity, fast signal response, wide linear range and low cost [8-15]. Therefore, it has been widely used in the study of phosphorylation and the detection of kinase activity, laying an important foundation for the research of kinase-related diseases and drugs. The phosphorylation of kinase requires the participation of phosphorylated donor ATP. Therefore, most electrochemical kinase biosensors rely on the modification and labeling of ATP, the change of electrical activity of phosphorylated substrates, or the introduction of ions and enzymes that can specifically recognize phosphate ions. Herein, we comprehensively review kinase-sensing methodologies during the past few years according to the types of recognition formats.

2.1 Interaction between substrate and kinase

The pre-designed peptide can form densely packed self-assembly monolayers (SAMs) on the electrode surface. The phosphorylation catalyzed by protein kinase can induce the change of the conformation and surface charge, and alter the electrostatic interaction toward the redox species in the solution. Therefore, the change of the electrochemical signal can be used to detect protein kinase activity and screen the inhibitors. For example, Li's group demonstrated that the positively charged SAM could prevent the positively charged $[Ru(NH_3)_5Cl]^{2+}$ getting close to the electrode due to the electrostatic repulsion [16]. However, the phosphorylation by kinase made SAM loose due to the electrostatic repulsion between the adjacent phosphate groups and the electrostatic attraction between the negative phosphate groups and positively charged SAM. Thus, $[Ru(NH_3)_5Cl]^{2+}$ molecules could get closer to the electrode surface and produce a significant electrochemical signal. The phosphorylation

of SAMs can also change the capacitance and interfacial electron transfer resistance (R_{CT}) at the electrode surface, and can also be investigated by electrochemical impedance spectroscopy (EIS) [17-21].

2.2 Labeled ATP as the substrate

ATP is usually as the donor of phosphate group in the phosphorylation process. In the presence of kinase, the phosphoric acid group at γ position can be transferred to the substrate. Therefore, the phosphoric acid group at γ position of ATP can be labeled by ferrocene (Fc), thiol or biotin group for the kinase-catalyzed phosphorylation. The labeled group can be transferred directly to the substrate. Fc is a widely used electroactive molecule and can be used to label and electrochemically "signal-on" monitor a specific phosphorylation event by cyclic voltammetry (CV). Since 2008, Kraatz's group has reported a series of works by employing the electroactive Fc-labeled ATP (Fc-ATP) as the co-substrate for electrochemical detection of kinase activity and screening of its inhibitors [22-26]. The schematic illustration of the electrochemical principle for protein kinase detection using Fc-ATP is shown in Figure 1A. In the presence of kinase and Fc-ATP, the substrate peptide was phosphorylated, and Fc group was brought into the serine/threonine or tyrosine residues of the substrate peptide, thus producing a detectable electrochemical signal to achieve direct and rapid detection [27]. They carefully investigated the influence of the linker in Fc-ATP conjugate (the length and hydrophily) on the performance of biosensors and found that a longer and more hydrophobic γ-Fc alkyl chain (C_6) outperformed the shorter and hydrophilic analogues as an efficient linker for kinase co-substrate[28-30].

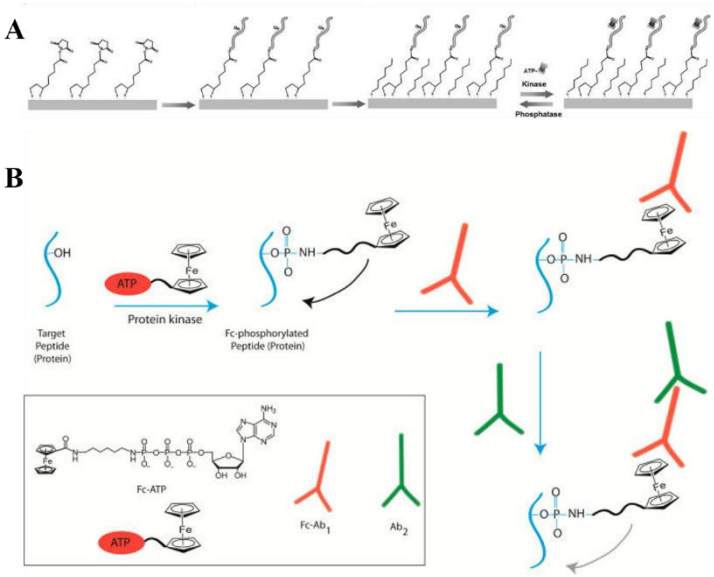

Figure 1. (A) Schematic illustration of the electrochemical principle for protein kinase detection using Fc-ATP. Reprinted with permission from [27]. Copyright 2008 American Chemical Society. (B) Strategy for Fc-phosphorylation of peptides and proteins. Target peptide or protein is Fc-phosphorylated in the presence of protein kinase and Fc-ATP cosubstrate. Reprinted with permission from [31]. Copyright 2012 American Chemical Society.

In addition, they also investigated the effect of Fc-phosphorylation type and sequence on the conformation and orientation of the surface-bound Fc-tau protein by using Fc-group as a signaling probe and a reporter. They developed a powerful and universal approach to monitor protein kinase activity by combining Fc-phosphoryl transfer with a robust polyclonal rabbit anti-Fc antibody (Fc-Ab_1) (Figure 1B) [31-33]. When the specific Fc-Ab_1 was added, the electrochemical signal-on response from the Fc-phosphorylated peptide was reduced. Furthermore, the fluorescent Ab_2 was used to study Fc-phosphoryl transfer on the surface via fluorescence approach.

5'-[γ-thio] triphosphate (ATP-S) can be employed as the co-substrate to capture the AuNPs based on the strong affinity between the thiophosphate and AuNPs, which has been used to develop various detection method, including electrochemical, ECL and fluorescence methods. In 2007, Kraatz's group for the first time reported the detection of kinase activity through labelling a specific phosphorylation reaction with ATP-S and AuNPs [34]. Then, monitoring the activity of protein kinase (protein kinase C (PKC) and protein tyrosine kinase (PTK)) *in vitro* was achieved by the voltammetric detection of AuNPs via Cl^- chemistry [35]. Wang's group developed an amplified electrochemical strategy for assay of protein kinase A (PKA) by using gold nanoparticles/multi-walled carbon nanotubes (AuNPs/MWNTs) nanohybrids to catalyze the 3, 3', 5, 5'-Tetramethylbenzidine (TMB) oxidation by H_2O_2 [36]. Estrela's group used on-chip multimodal sensing approach to screen kinase inhibitors [37, 38]. After AuNPs was captured by thiols in peptide, they added thiolated-Fc to modify AuNPs, allowing for detection of phosphorylation using DPV and EIS. Liu's group also reported a sensitive electrochemical strategy for the detection of kinase activity based on the 4-mercaptophenylboronic acid (MPBA)-induced *in situ* assembly of silver nanoparticles [39]. In the study, after phosphorylation with ATP-S as the co-substrate, citrate-capped silver nanoparticles (AgNPs) were conjugated specifically to the thiophosphate peptides via the Ag-S interactions. Then, the MPBA molecules present in solution could further induce the assembly of AgNPs into network-like structure through the Au-S and citrate-boronate interactions, thus producing the amplified electrochemical signal. Meanwhile, AuNPs have also been widely used as a sensitive signal transduction probe to construct ECL biosensors. To improve the sensitivity of kinase activity analysis, Li's group first applied AuNPs to mediate the ECL reaction of ECL indicators and amplify the ECL signal [40]. As shown in Figure 2A, the dense and insulated peptide layer hampers the electron transfer between luminol and electrode. However, after the phosphorylation reaction with PKA and ATP-S, the bounded AuNPs on the electrode surface by the formation of Au-S bonds can hugely amplify the ECL signal from luminol. With the similar strategy, Qiu's group designed an ECL biosensor for highly sensitive detection of protein kinase activity by employing ATP-S and AuNPs [41, 42]. Recently, Ai's group described a photoelectrochemical (PEC) method to detect PKA activity based on the Cd-S interaction between the CdS quantum dots (QDs) and thiol groups (Figure 2B) [43]. In this work, the peptide was immobilized on the hybrid of graphite-like carbon nitride (g-C_3N_4) and AuNPs (g-C_3N_4-AuNPs). After the phosphorylation by PKA in the presence of ATP-S, CdS QDs was bound to the electrode to increase the PEC response of g-C_3N_4.

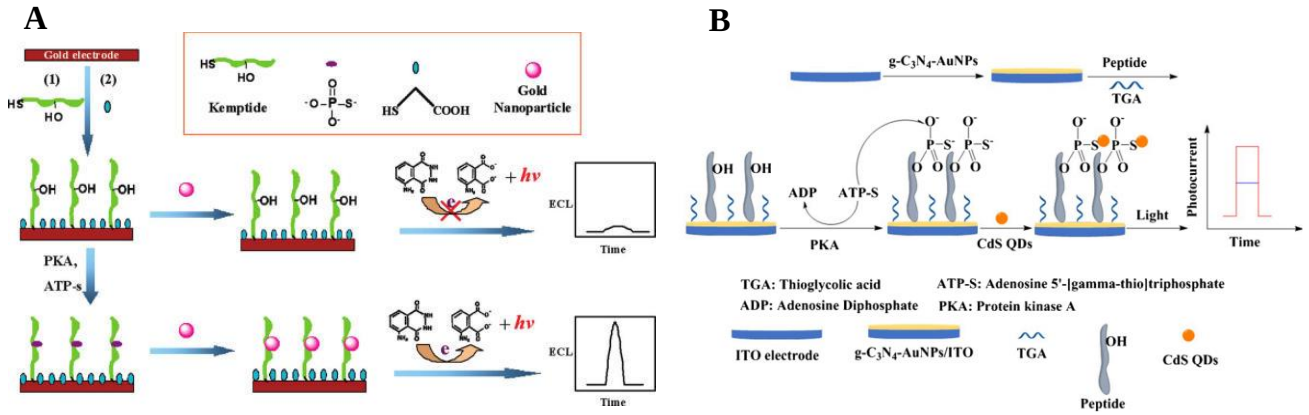

Figure 2. (A) Schematic representation of ECL strategy for kinase activity detection using gold nanoparticles as signal transduction probes. Reprinted with permission from [40]. Copyright 2010 American Chemical Society. (B) Schematic illustration of the fabrication process of the electrode and PEC strategy for PKA activity assay. Reprinted with permission from [43]. Copyright 2018 Springer.

Biotin-modified ATP (ATP-biotin) is a ubiquitous accepted as a co-substrate. After kinase-catalyzed biotinylation with ATP-biotin, the biotin group facilitated the analysis of phosphoproteins with various streptavidin-conjugated reagents or nanomaterials [44-48]. For example, Kerman's group demonstrated an electrochemical method for protein-phosphorylation detection based on the biotinylation of the kinase substrate with ATP-biotin as the co-substrate [49]. After the accomplishment of the biotinylation of the peptide, streptavidin-coated Au nanoparticles were introduced and conjugated to the biotinylated peptide through the high affinity between the streptavidin and biotin. Then, the kinase activity was monitored by detecting the reduction of tetrachloroaurate ions produced from the electrochemical oxidation of AuNPs in 0.1 M HCl medium.

2.3 Tyrosine oxidation

It has been suggested that phenolic hydroxyl groups, tryptophan and its residues in proteins can be electro-oxidized at carbon electrodes involving a two-electron and two-proton transfer [50-52]. However, when the hydroxyl group of Tyr is phosphorylated into a phosphate group (Tyr-P), the electrocatalytic activity was suppressed and caused the shift of the oxidation current to a higher oxidation potential than that of the unmodified residue, which has been used to electrochemically detect the phosphorylation process and other clinically important proteins and peptides. Based on this principle, many researchers designed electrochemical biosensors to detect tyrosine kinase activity. For example, Kerman's group presented a label-free electrical sensing of small-molecule inhibitors on tyrosine phosphorylation based on the different responses of phosphorylated and non-phosphorylated tyrosine residues (Figure 3A) [53]. Before the phosphorylation, Tyr oxidation was achieved at ~0.65 V using a MWCNT-modified screen-printed carbon electrodes (SPCE). After the phosphorylation with ATP, Tyr-P can not be oxidized and the current response is suppressed, or probably shifted to a higher peak potential. However, once a small-molecule inhibitor was added, the phosphorylation is successfully inhibited and Tyr can be oxidized, leading to a high current response. Based on the same

principle, Guo's group developed a label-free electrochemical biosensor for the differentiation of phosphorylated and non-phosphorylated peptide as well as the protein tyrosine kinase activity and inhibition by using $Os(bpy)_3^{2+}$ (bpy = 2,2'-bipyridine) as an electron mediator to catalyze the oxidation of tyrosine [54, 55]. Einaga's group investigated the electrochemical oxidation behaviors of Tyr, Tyr-P and Tyr-S using boron-doped diamond (BDD) electrodes [56].

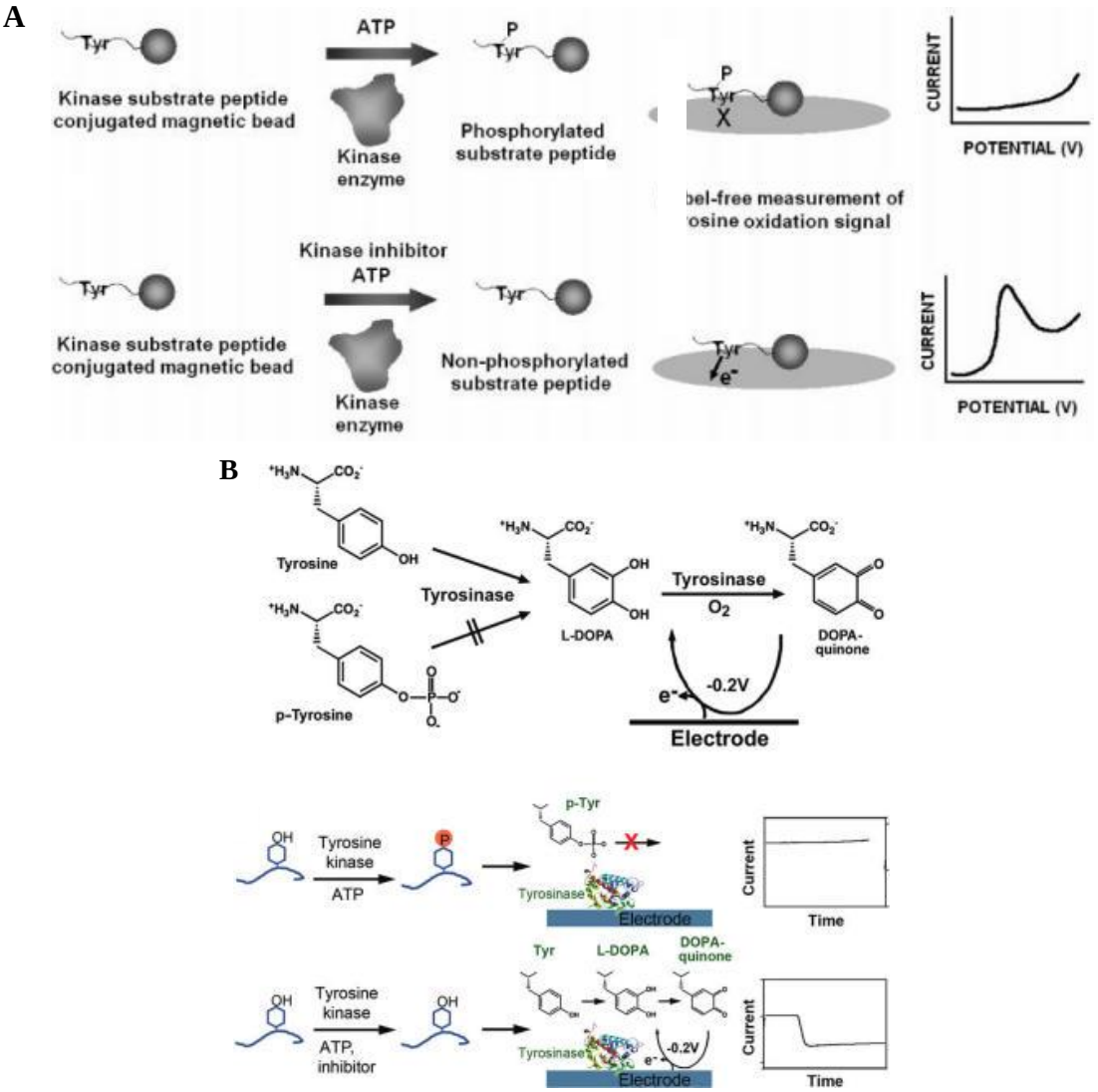

Figure 3. (A) Schematic representation of the principle for the tyrosine (Tyr) oxidation-based detection of protein phosphorylation. Reprinted with permission from [53]. Copyright 2007 American Chemical Society. (B) Working model for the determination of the activity of protein tyrosine kinases and schematic illustration of the principal for tryosinase-based detection of the phosphorylation of peptide. Reprinted with permission from [57]. Copyright 2012 American Chemical Society.

To avoid the necessary to immobilize the peptide on the electrode, Yuan's group reported a reusable amperometric biosensor for measuring PTK (p60c-src Protein tyrosine kinase (Src)) activity by using the enzyme (tyrosinase) electrode (Figure 3B).[57] In this work, tyrosinase could catalyze the oxidation of Tyr to L-DOPA (L-3,4-dihydroxyphenylalanine) and L-DOPA-quinone. Next, the L-DOPA-quinone was electrochemically reduced to L-DOPA at −0.2 V. The Src-catalyzed phosphorylation reaction of peptide suppressed the tyrosinase-catalyzed oxidation of Tyr. Therefore,

Src activity and inhibitor could be sensitively measured repetitively without regeneration. Xian's group also developed an immobilization-free biosensor for the activity assay of PTK by using a graphene modified glassy carbon electrode, in which graphene enhanced the electrochemical response of Tyr through electrocatalytic oxidation reaction [58]. To amplify the electrochemical signal and improve the sensitivity, Liu's group integrated the tyrosinase-catalyzed oxidation of Tyr with the MPBA-induced *in situ* assembly of AgNPs for the detection of protein kinase activity [39]. In this strategy, L-DOPA, the product of tyrosinase-catalyzed oxidation, reacted with MPBA through the formation of boronate ester bonds, and subsequently conjugated with AgNPs *via* the Ag-S interactions. The MPBA molecules initiated the assembly of citrate-capped AgNPs into network-like structure. However, once Tyr residues were phosphorylated by PTK, the phosphorylated peptides could not be oxidized by tyrosinase and induce the formation of AgNPs aggregates, resulting in a sharp decreased signal.

2.4 Interaction between metal ion and phosphate

It has been suggested that many metal ions (such as Zr^{4+}, Ti^{4+}, Fe^{2+}, Zn^{2+} and Ag^{+}) and metal oxides (such as ZrO_2 and TiO_2) can specifically bind to phosphate group of phosphorylated substrate. Based on this property, these metal ions, metal complexes, and metal oxides have been successfully used in separation and enrichment of peptide, and also provide a new idea for the detection and analysis of kinase activity. TiO_2 NPs have been used to isolate phosphopeptides from complex bio-samples through the interaction between the phosphate functional groups and the surface of TiO_2 particles. TiO_2 NPs can also be applied to develop electrochemical biosensors for protein kinase activity in view of the large number of Ti^{4+} ions on the surface. For example, Liu's group reported a TiO_2-assisted silver enhanced biosensor for kinase activity profiling [59]. In this study, after the PKA-catalyzed reaction in the presence of ATP, the phosphorylated serine residues of Kemptides were specifically labeled by TiO_2 NPs. Then, Ag^{+} was added and then photocatalytically reduced to AgNPs by the excited electrons of TiO_2 NPs under UV light. Finally, the quantity of the phosphorylated peptides could be detected by employing AgNPs as the detectors by DPV. TiO_2 NPs-based composites can also be used as the electrode substrate to immobilize the peptide [60]. On the other hand, TiO_2 NPs can act as a sensitizer due to the high activity under UV excitation. Ai's group used the g-C_3N_4 and TiO_2 complex to capture the phosphorylated peptide (P-peptide) to PEC detection of PKA activity (Figure 4) [61]. Then, the assembly of P-peptide, PAMAM dendrimer and alkaline phosphatase (ALP) was formed on the surface of the electrode to produce an increased photocurrent. They also applied AuNPs decorated zeolitic imidazolate frameworks as the electrode substrate and the g-C_3N_4 and TiO_2 complex as recognition and photochemical label for PKA detection. Besides, titanium ion (Ti^{4+}) was used as a specific mediator for the ligation of two phosphate groups to develop a sensitive electrochemical biosensor for the detection of the T4PNK activity by Wang and co-workers [62]. Capture probe DNA was covalently modified on the surface of gold electrode. In the presence of T4PNK, the 5'-hydroxyl end of captured DNA was phosphorylated. With Ti^{4+} as the linker, a single-walled carbon nanotube (SWCNT) modified with phosphorylated DNA and ferrocene was assembled on the electrode surface to generate an electrochemical signal. The detection limit of the method for T4PNK is 0.01 U/mL.

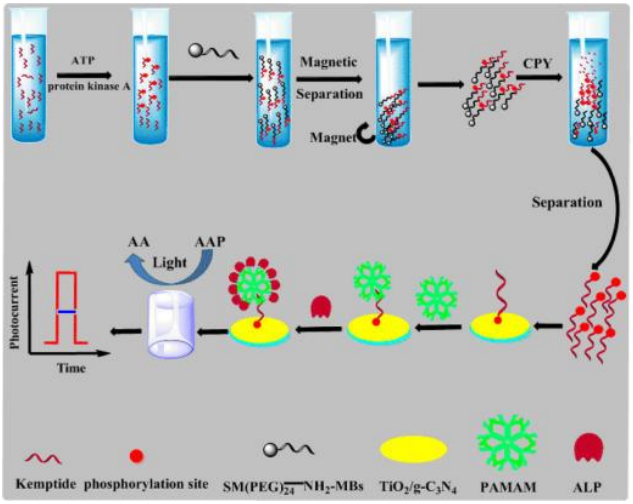

Figure 4. Schematic representation of the fabricated PEC biosensor for assay of PKA activity based on PKA-catalyzed phosphorylation reaction in solution and signal amplification of TiO_2/g-C_3N_4, PAMAM-COOH, and ALP. Reprinted with permission from [61]. Copyright 2017 American Chemical Society.

The multi-coordinative interactions between Zr^{4+} and phosphate group on the phosphorylated peptide have made Zr^{4+} widely be used in designing of kinase activity biosensors, especially by combination with different nanomaterials [63-67]. Zr^{4+} was employed as the "signal reporter" and the phosphorylation indentifier to link phosphate group in the phosphorylated peptide. In 2009, Nie's group first presented a Zr^{4+}-based electrochemical strategy for label-free monitoring of the activity and inhibition of protein kinase [68]. In their strategy, DNA functionalized AuNPs (DNA-AuNPs) are utilized as the "signal reporter" because of the intrinsic 5'-phosphate end of DNA and the excellent properties of DNA-AuNPs in electrochemical signal generation and amplification. The peptide phosphorylation catalyzed by PKA induced the attachment of AuNPs on the surface of the electrode. Then, a large number of electroactive molecules $[Ru(NH_3)_6]^{3+}$ (RuHex) were electrostatically loaded on the DNA–AuNPs with negative charges and the chronocoulometric response of RuHex were monitored for the detection of the PKA activity. To further amplify the current signal, Li's group integrated the DNA assembly induced AuNPs polymeric network into this strategy (Figure 5A) [69]. Two sets of AuNPs were modified with DNA_1 and its complementary sequence DNA_2, respectively. Once DNA-AuNPs was captured by the P-peptide, the hybridization between DNA_1 and DNA_2 would induce DNA-AuNPs to assemble into polymeric network block, finally generating the significantly amplified amperometric responses. Li's group also developed a novel electrochemical method for sensing protein phosphorylation based on the Zr^{4+}-mediated signal transition and the signal amplification of rolling circle amplification (RCA) reaction (Figure 5B) [70].

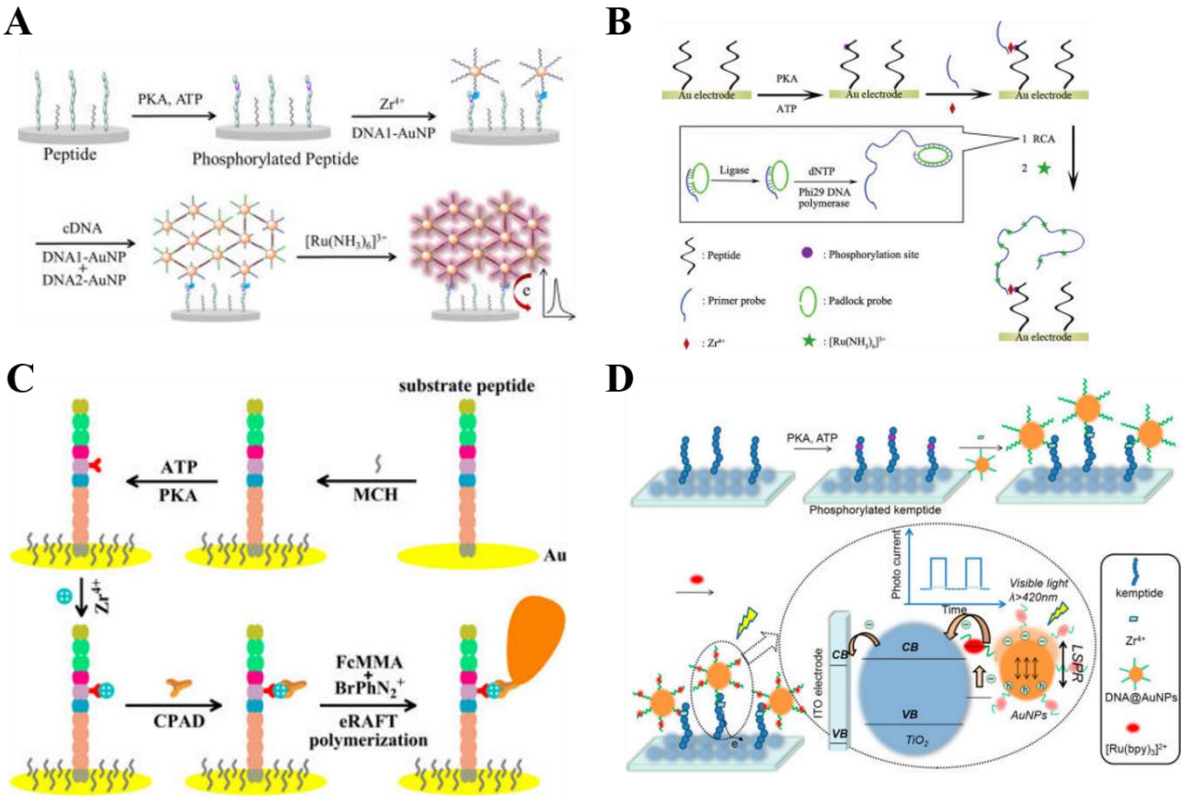

Figure 5. (A) Configuration of DNA-AuNPs assembled polymeric network amplified electrochemical biosensor for kinase activity detection. Reprinted with permission from [69]. Copyright 2014 American Chemical Society. (B) Schematic illustration of the strategy for monitoring the activity of protein kinase based on Zr^{4+} mediated signal transition and the signal amplification by RCA reaction. Reprinted with permission from [70]. Copyright 2012 American Chemical Society. (C) Fabrication of the eRAFT-polymerization-based electrochemical biosensor. Reprinted with permission from [71]. Copyright 2019 American Chemical Society. (D) Configuration of photoelectrochemical biosensor for kinase activity setection. Reprinted with permission from [72]. Copyright 2016 American Chemical Society.

The linkage of phosphorylated peptide and DNA primer probe by Zr^{4+} would initiate RCA with the aid of the padlock probe and phi29 DNA polymerase on the surface of the electrode. The product with a long DNA strand absorbed a large number of RuHex near the electrode surface *via* the electrostatic interaction, and thus cascade electrochemical signals were obtained. The *in situ* electrochemically mediated grafting of electroactive polymers from the phosphorylated sites can also be used to design effective signal amplification strategy [73]. For instance, recently, Niu's group reported the electrochemically controlled reversible addition-fragmentation chain transfer polymerization as a novel amplification strategy for PKA sensing (Figure 5C) [71]. In this method, Zr^{4+} was anchored on the carboxyl group-contained chain transfer agents to the phosphorylated sites of the peptide on the gold electrode. Once the polymerization was initiated under a potentiostatic condition, numerous electroactive Fc tags contained in the monomer ferrocenylmethyl methacrylate (FcMMA) can be linked to each phosphorylated site, leading to a significant amplification of the electrochemical signal. Besides, Ru(II) encapsulated phosphorylate terminated silica nanoparticles, the xanthine oxidase and DNA conjugated AuNPs. Zr-based metal-organic framework with porphyrin

groups (MOF-525-Zn) were utilized as multiple signal amplification nanoprobes to develop a novel ECL biosensor for the detection of PKAs [74-76]. A dye-sensitized and localized surface plasmon resonance (LSPR) enhanced visible-light photoelectrochemical biosensor was also developed by Liu's group for PKA analysis [72]. As shown in Figure 5D, through the coordination between Zr^{4+} and phosphate groups, DNA-AuNPs were assembled on the P-peptide modified TiO_2/ITO electrode. $[Ru(bpy)_3]^{2+}$ was intercalated into the grooves of DNA on AuNPs. Then, under the visible light, $[Ru(bpy)_3]^{2+}$ injected excited electrons into the TiO_2 conduction band to generate an improved photocurrent, due to LSPR signal amplification and dye sensitization. The detection limit of this PEC biosensor was 0.005 U mL^{-1} (*S/N* = 3).

Molecules with a bis(Zn^{2+}-dipicolylamine) moiety can specifically recognize the phosphate monoester dianion in phosphorylated peptides or proteins at neutral pH [77]. For example, Hong's group constructed a homogeneous assay for PKA where synthetic receptors were immobilized on an electrode surface and cAMP-dependent protein kinase catalyzed the phosphorylation of its specific peptide in aqueous media (Figure 6) [78]. The synthetic receptor consisted of a bis(Zn^{2+}-dipicolylamine) moiety with a higher affinity toward the phosphorylated peptide and a thiol group at the other end to self-assemble onto the surface of the gold electrode. The peptide substrate was phosphorylated by the kinase and was then bound to the surface through the binuclear Zn^{2+} complex, generating an electrochemically oxidative signal from the conjugated Fc group. They also employed AuNPs as the bridge between the electrode and bis(Zn^{2+}-dipicolylamine) moiety to facilitate the oxidation of Fc and enhance the electrochemical signal [79]. As a kind of commercially specific phosphate-binding reagent, the Phos-tag, containing the dinuclear zinc(II) complex, can further functionalized with biotin, which has great potential in protein kinase activity assay [80-82]. For example, Ai's group utilized the biotin-functionalized Phos-tag (Phos-tag-biotin) to recognize P-peptide [83]. Next, avidin-functionalized horseradish peroxidase (HRP) was captured on the electrode surface through the biotin-avidin interaction. Therefore, the PKA activity and inhibitors were determined by monitoring the electrochemical reduction current of the oxidized benzoquinone by H_2O_2 under the catalytic effect of HRP. For multiple signal amplification, they applied functional nanomaterials (including AuNPs@carbon nanospheres hybrid material (Au@C), SiO_2-strepavidin nano-composite (SiO_2-SA), AuNPs-biotin-β-galactosidase (Au-B-Gal)) to develop a sensitive electrochemical biosensor for PKA activity [84]. A large number of β-galactosidases could catalyze the hydrolysis reaction of p-aminophenyl galactopyranoside (PAPG) to p-aminophenol (PAP), which generated the electrochemical signal to detect the phosphorylation level of peptide and PKA activity. Ai's group also used the specific recognition ability of Phos-tag to design a PEC biosensor for protein kinase activity assay based on visible-light active g-C_3N_4-AuNPs as the electrode substrate [85].

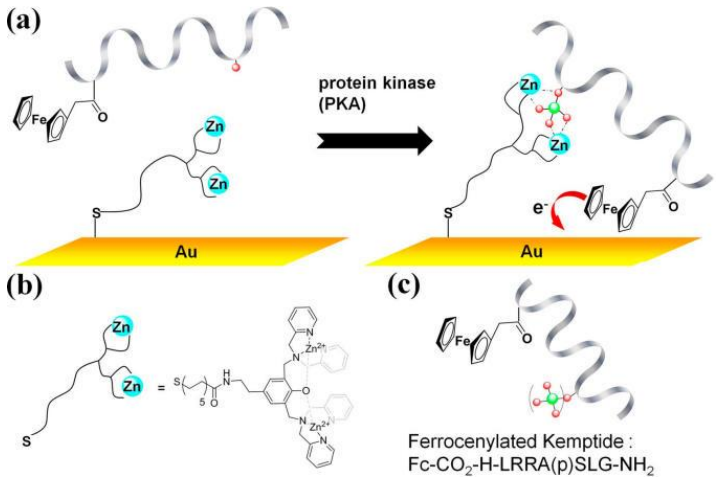

Figure 6. (a) Schematic strategy of homogeneous electrochemical assay for protein kinase activity, (b) structure of the phosphorylated peptidebinding receptor, and (c) ferrocenylated kemptide was used as a peptide substrate for PKA. Reprinted with permission from [78]. Copyright 2014 American Chemical Society.

Like the Ti^{4+}- and Zr^{4+}-mediated conjugation of phosphate group-contained signal probe and phosphorylated substrate peptide, Fe^{3+} also exhibits high affinity to the kinase-generated phosphorylated sites of peptides. Meanwhile, Fe^{3+} shows the high electrocatalytic activity toward the reduction of H_2O_2. Xia's group developed a label-free and sensitive electrochemical biosensor to monitoring protein kinase activity and inhibition based on the electrocatalytic activity of Fe^{3+} [86]. In this method, Fe^{3+} was captured by the phosphorylated peptide based on the interaction of Fe^{2+} and phosphate group. The captured Fe^{3+} can catalyze the electrochemical reaction of H_2O_2 to produce electrical signal. The detection limit of this method for PKA is 0.1 U/mL. Wang's group further improved the sensitivity by numerous Fe^{3+}-immobilized AuNPs to recognize phosphorylated sites and catalyze H_2O_2 redox [87]. Willner's group presented that Ag^+ can be absorbed by the phosphorylated monolayer-modified electrode and be electrochemically reduced on electrode surface.[88]

Besides, Cao group reported that the phosphorylation site in P-peptide can also be recognized by arginine (Arg) residues in peptide [89]. In the work, the artificial peptide P2 with arginine residues contains a short template for the preparation of copper nanoparticles (P2-CuNPs). When the PKA-specific peptide P1 modified on the electrode was phosphorylated with catalysis of PKA, P2-CuNPs would bind with P2-CuNPs through ultra-stable phosphate-guanidine interaction and the amplified electrochemical response is produced from dissolution of surface-attached CuNPs.

2.5 Enzymatic cleavage

Because of the specific recognition and different cleavage efficiency of peptide before and after phosphorylation, electrochemical kinase biosensors can be designed based on the enzymatic cleavage of substrate. For example, Yin and co-workers reported the electrochemical detection of casein kinase II (CK2) activity by the selective cleavage of carboxypeptidase Y (CPY) to substrate peptide [90]. In the absence of CK2, CPY induced the hydrolysis of the substrate peptide modified on the electrode surface into free amino acid fragments, and the exposure of electrode facilitated the electron transfer of

$Fe(CN)_6^{3-}$ in the solution to the electrode surface. In the presence of CK2, the phosphorylation of the substrate peptide can inhibit the hydrolysis of CPY. Therefore, $Fe(CN)_6^{3-}$ cannot be effectively close the electrode surface, resulting in a low electrical signal. CK2 at the concentration of 0.041 U/mL was readily determined. To avoid the disadvantage of the labeling of the peptide, ATP and the electrode, Wang's group presented a label-free homogeneous electrochemical strategy for protein kinase assay based on CPY-assisted peptide cleavage reaction and vertically ordered mesoporous silica films (MSFs) (Figure 7) [91]. The artificially synthesized peptide consisted of a kinase-specific recognized sequence and a multiple positively charged arginine (R) residues-rich sequence. The peptide was phosphorylated by kinase and resisted the cleavage by CPY. Then, the phosphorylated peptide electrostatically absorbed on the surface of MSFs-modified ITO electrode (MSFs/ITO), preventing the diffusion of the electroactive probe (FcMeOH). On the contrary, without kinase, the peptide was digested into free amino acids. Thus, numerous free FcMeOH in solution easily diffused toward the underlying electrode surface, resulting in an increased electrochemical signal. In this sensing platform, The detection limits of the method for PK2 and CK2 were 0.083 and 0.095 U/mL, respectively.

Figure 7. Schematic diagram for the label-free homogeneous electrochemical sensing platform for protein kinase assay based on CPY-assisted peptide cleavage and vertically ordered MSFs. Reprinted with permission from [91]. Copyright 2017 American Chemical Society.

Polynucleotide kinase (PNK) can catalyze the phosphorylation of nucleic acids with 5'-hydroxyl termini, and initiate the hydrolysis of the 5'-phosphoryl termini catalyzed by λ exonuclease (λ exo). Li's group have developed a simple dual-signal electrochemical biosensor for the detection of T4 polynucleotide kinase (PNK) based-on λ exo-mediated cleavage of phosphate group at the 5'-terminal of dsDNA [92]. Firstly, a DNA probe with G-rich bases was covalently cross-linked to the electrode surface for the capture of Fc-labeled DNA. The Fc molecule was close to the electrode and thus produced a strong electrical signal. In the presence of PNK, the 5'-hydroxyl end of DNA was phosphorylated and then cleaved by λexo, which made Fc far away from the electrode surface, thus resulting in a decrease in the electrical signal. At the same time, the captured DNA on the electrode surface can form G-tetramer to catalyze the electrochemical reaction of H_2O_2, which led to an enhanced electrical signal. With the change of these two signals, PNK at a minimum of 0.02 U/mL was readily detected. Zhang's group reported a new electrochemical biosensor for the detection of

PNK based on the enhanced quasi-reversible redox signal of prussian blue (PB) generated by self-sacrificial label of iron metal-organic framework (FeMOF) [93]. In this study, AuNPs modified FeMOF (FeMOF@AuNPs) was employed to immobilize DNA hairpin probe. After the phosphorylation by PNK and the digestion by λexo, the produced FeMOF@AuNPs-labeled ssDNA would hybridize with the capture DNA on the electrode surface. Fe^{3+} in the MOF reacted with $K_4Fe(CN)_6$ to produce PB on the electrode surface, consequently generating a high electrochemical signal. Recently, Zhang's group also designed a sensitive amperometric assay for the detection of the activity of PNK based on the mimetic catalysis of DNA functionalized Fe-porphyrinic MOF ($(Fe\text{-}P)_n$-MOF) and enzyme-assisted amplification reaction (Figure 8) [94]. In this research, the trigger was released from the phosphorylation by PNK and the subsequent digestion by λexo, and initiated the enzyme-assisted amplification reaction with the aid of restriction endonuclease Nb. BbvCI on the electrode surface. Then $(Fe\text{-}P)_n$-MOF was bound to the electrode and peroxidase-like catalyzed the oxidation of TMB in the presence of H_2O_2, producing the chronoamperometric signal.

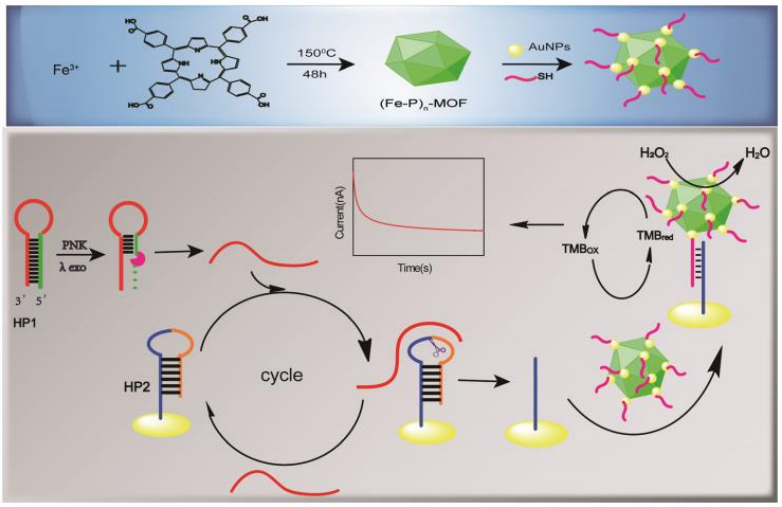

Figure 8. Schematic illustration of the preparation procedure of L/(Fe-P)n-MOF probe and the electrochemical detection strategy for T4 PNK activity. Reprinted with permission from [94]. Copyright 2019 Springer.

2.4 Recognition of P-peptide by antibody and protein

Although ATP-labeled biosensors can directly and effectively determine kinases, they require the separation and purification process of ATP synthesis, which is time-consuming and cumbersome and may affect the activity of ATP. Thus, their practical applications are still limited. P-peptide can be specifically recognized by the anti-P-peptide antibody. In 2008, Willner' group demonstrated that after the phosphorylation of the peptide, the anti-P-peptide antibody can bind with P-peptide and insulate the electrode surface, further increasing the interfacial electron-transfer resistance (Figure 9A) [95]. Furthermore, the anti-P-peptide antibody has been modified with biotin to link other functional biomolecules or NPs *via* the biotin–avidin interaction [98, 99]. When the P-peptide was biotinylated by the anti-P-peptide antibody, streptavidin and HRP-loaded AuNPs were immobilized on the surface of the electrode to catalyze the oxidation of TMB, generating an electrochemical signal for monitoring

phosphorylation [100]. Ai's group also developed an amperometric platform for the assay of kinase activity based on HRP-conjugated AuNPs (Figure 9A) [96]. In their method, HRP-conjugated IgG (IgG-HRP) functionalized AuNPs were captured on the surface of the electrode *via* the specific immunoreaction between anti-phosphoserine antibody and HRP-IgG. HRP can effective catalyze the oxidization of hydroquinone in the presence of H_2O_2, which could be electrochemically reduced and generated a sensitive electrochemical reduction signal. An ECL bioassay for two kinases based on the recognition of the P-peptide by the anti-P-peptide antibody is reported by Qi's and co-workers. As shown in Figure 9B, a ruthenium derivative-labeled protein A was utilized as a versatile ECL probe and specifically bound to the anti-P-peptide antibody on the electrode surface, producing an ECL emission in the presence of tripropylamine (TPA).

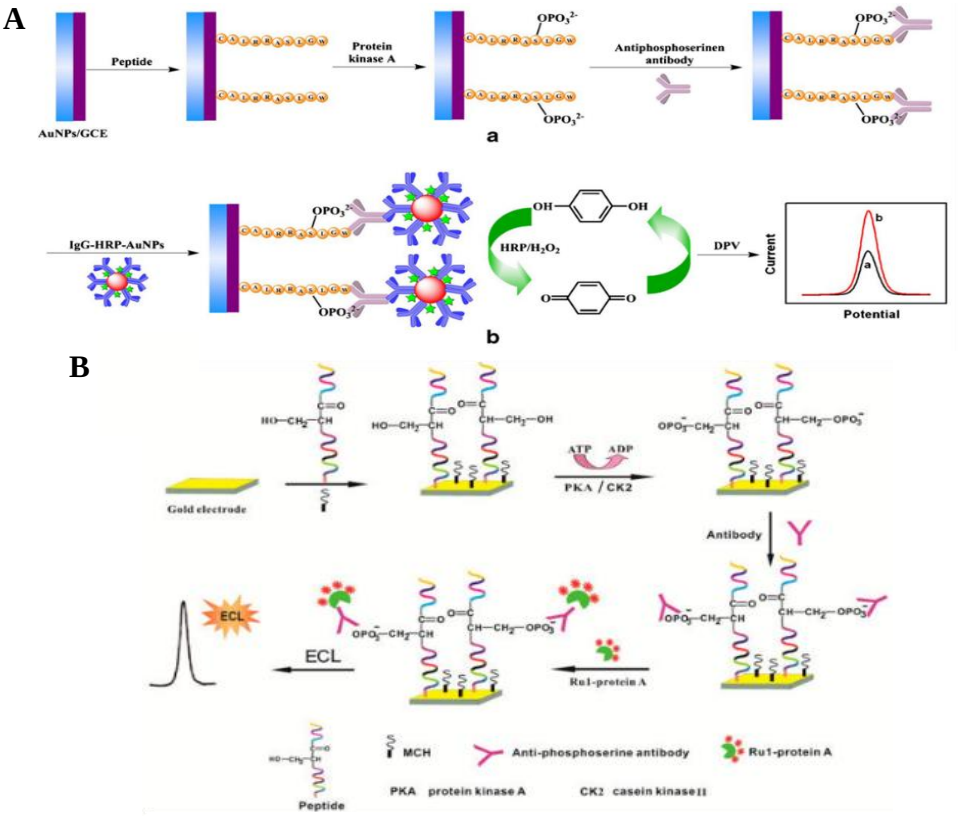

Figure 9. (A) Analysis and amplification of casein kinase activity by electrochemical impedance spectroscopy. Reprinted with permission from [95]. Copyright 2008 Wiley-VCH Verlag GmbH& Co. (B) Schematic illustration of the fabrication and PK activity detection process of the electrochemical immunosensor based on HRP-conjugated AuNPs. Reprinted with permission from [96]. Copyright 2017 Springer-Verlag Wien. (C) Schematic diagram of ECL bioassay of protein kinases. Reprinted with permission from [97]. Copyright 2016 American Chemical Society.

2.7 Interaction between kinase and antibody, aptamer or peptide

The specific interaction between kinase and the complementary aptamer can induce a structure transition of aptamer and the change of the charge property of the surface [101]. Based on this strategy, Miao's group presented an ultrasensitive and simple method for protein tyrosine kinase-7 (PTK7)

detection [102]. In the absence of PTK7, DNA-AuNPs was captured on the electrode surface by hybridizing with the aptamer and helper probe. Then, abundant methylene blue (MB) molecules were captured by both the sandwiched dsDNA complex and the AuNPs-modified ssDNAs, leading a strong current signal. The presence of PTK7 would cause a structure change of the aptamer, hamper the binding of DNA-AuNPs and the following capture of MB. Kim and co-workers also described a polymeric microchip electrolyte-insulator-semiconductor sensing in a Teflon cell for the capacitive detection of PKA [103]. First, the AuNPs decorated sensor surface was modified with the thiolated PKA-specific aptamer. Then, the aptamer-PKA interaction would cause the change in local surface charge.

Receptor tyrosine kinase AXL plays an important role in the pathophysiology of heart failure (HF). Asadpour-Zeynali and co-workers developed an electrochemical immunosensor for receptor tyrosine kinase AXL using poly(pyrrolepropionic acid)-modified SPCEs [104]. A specific capture antibody was covalently immobilized on the modified screen-printed carbon electrodes. When AXL was captured, the immune-sandwich complex formed with a biotinylated detector antibody labeled with a streptavidin-HRP conjugate. Upon the addition of H_2O_2, AXL was determined through measuring the amperometric responses of the HRP-catalyzed oxidation of hydroquinone. Based on the same strategy, they utilized amine functionalized graphene quantum dots (fGQDs) to modify SPCE and monitored the affinity reactions by measuring the decrease in the DPV response of the redox probe $Fe(CN)_6^{3+/4+}$ [105].

Some peptide analogs of the recognition motifs in protein kinases are good kinase inhibitors through the strong protein-peptide interaction. For example, PKA inhibitor containing an RRNAI motif can bind with PKA and thus can inhibit its activity. Sun and co-workers found that this peptide inhibitor could interact with the negatively charged citrate-capped AuNPs and further induce the formation of the aggregation of AuNPs (Figure 10) [106]. However, when it specifically combined with PKA, the peptide can not induce the aggregation any more. They converted AuNPs-based colorimetric assay into sensitive electrochemical analysis of protein kinase. The synthetic peptide inhibitor (IP_{20}) immobilized on the electrode can attach AuNPs. Interestingly, AuNPs can recruit more Fc-peptide and AuNPs through the electrostatic interaction, thus resulting in the formation of AuNPs-peptide-AuNPs network architecture on the electrode surface and leading to a significant decrease in the charge transfer resistance. However, when IP_{20} interacted with PKA, it can not induce the formation of the network of AuNPs.

2.8 Other methods

The abovementioned methods are feasible but require the synthesis and immobilization of a specific peptide substrate, which is expensive and vulnerable to environmental influence. Thus, it will be interested to develop peptide-free and sensitive method to determine the activity of kinase and its inhibitors. Yang's group reported a single electrochemical biosensor for detecting the activity and inhibition of both protein kinase and alkaline phosphatase. Specifically, PKA and ALP can promote the hydrolysis of ATP and pyrophosphate, respectively [107]. Both the two enzyme-catalyzed reactions cause the production of phosphate ions, which is proportional to the corresponding enzyme

activity. Then, the produced phosphate ions reacted with molybdate to form redox molybdophosphate precipitates on the electrode surface, producing a strong electrochemical signal. Wang's group developed an electrochemical method for assay of kinase activity by coupling the ATP-aptamer interaction with hybridization chain reaction (HCR) [107]. In the presence of PKA, ATP is hydrolyzed into ADP that can not bind with the ATP aptamer. The aptamer hybridized with the capture probe and triggered HCR between two complementary hairpin DNA (H1, H2), yielding nicked double-helices DNA polymer. Then, MBs were intercalated into the grooves of the dsDNA polymers and produced an amplified electrochemical signal. However, in the absence of PKA, ATP would preferentially bind to the aptamer with high affinity and the HCR on the sensing surface was hampered, thus leading to a significantly decrease in the electrochemical signal.

4. CONCLUSION

Abnormal expression of kinases will affect phosphorylation in human body, which is related to many diseases, such as cancer, Alzheimer's disease and diabetes mellitus. The electrochemical biosensor has the advantages of simple operation, high sensitivity, fast analysis speed, relatively low cost, easy miniaturization and integration. In particular, the introduction of signal amplification technology into the construction of electrochemical biosensors is of great help to improve the sensitivity and specificity of electrochemical biosensors and enhance their detection signals.

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