Talanta 206 (2020) 120173

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Ultrasensitive peptide-based electrochemical detection of protein kinase activity amplified by RAFT polymerization

Qiong Hu[a,b], Jinming Kong[c,**], Dongxue Han[a,b], Yu Bao[a], Xueji Zhang[d,***], Yuwei Zhang[a,*], Li Niu[a,b]

[a] Center for Advanced Analytical Science, School of Chemistry and Chemical Engineering, Guangzhou University, Guangzhou, 510006, PR China
[b] School of Civil Engineering, Guangzhou University, Guangzhou, 510006, PR China
[c] School of Environmental and Biological Engineering, Nanjing University of Science and Technology, Nanjing, 210094, PR China
[d] Research Center for Bioengineering and Sensing Technology, School of Chemistry and Biological Engineering, University of Science and Technology Beijing, Beijing, 100083, PR China

ARTICLE INFO

Keywords:
Electrochemical biosensor
Protein kinase
Signal amplification
Substrate peptide
Inhibitor screening
RAFT polymerization

ABSTRACT

Since the oversecretion of protein kinases is indicative of multiple human diseases, the screening of their activities is quite important to clinical diagnosis and targeted therapy. In this work, an ultrasensitive peptide-based electrochemical biosensor was presented for the detection of protein kinase activity by using the reversible addition-fragmentation chain transfer (RAFT) polymerization technique as a signal amplification strategy. First, the substrate peptides were tethered to a gold electrode surface via the thiol terminals. After the phosphorylation of substrate peptides by protein kinases, the carboxyl group-containing dithiobenzoates were labeled to the phosphorylated sites via the robust phosphate-Zr^{4+}-carboxylate linkages. Finally, the RAFT polymerization was initiated using ferrocenylmethyl methacrylates (FcMMAs) and dithiobenzoates as the monomers and the RAFT agents, respectively. The grafting of ferrocenyl polymer chains efficiently recruits a great number of electroactive Fc probes to each phosphorylated site, leading to a drastic amplification of the electrochemical signal. With PKA (protein kinase A) as the target, the detection limit of the peptide-based biosensor can be as low as 1.05 mU mL^{-1}. Moreover, it can selectively differentiate the target from other interferents and is applicable for the screening of potential inhibitors as well as the detection of protein kinase activity in complex cell lysates. Therefore, the peptide-based biosensor shows great promise as a universal tool for protein kinase activity detection and inhibitor screening.

1. Introduction

Using adenosine 5′-triphosphate (ATP) as the co-substrate (as the phosphate group donor), protein kinases such as the cAMP-dependent protein kinase (PKA) can site-specifically phosphorylate the hydroxyl groups of tyrosine (Tyr), threonine (Thr), or serine (Ser) residues in a protein or peptide substrate [1]. Phosphorylation of proteins is an essential post-translational modification (PTM) and is pivotal to signal transduction (e.g., immunity) and cellular regulation (e.g., cell proliferation) [2–4]. Because the oversecretion of protein kinases is indicative of multiple human diseases (e.g., diabetes, cardiovascular diseases, Alzheimer's disease, and cancers) [3,5,6], the screening of protein kinase activity is greatly important to protein kinase-related disease diagnosis and targeted therapy.

Currently, an array of peptide-based methods, such as radiometric [7], resonance light scattering (RLS) [8], photoelectrochemical (PEC) [9], electrogenerated chemiluminescent (ECL) [10], quartz-crystal microbalance (QCM)-based [11], fluorometric [12,13], colorimetric [14,15], and electrochemical [16–20], have been presented for the detection of protein kinase activity. Among them, electrochemical methods have drawn ever-increasing attention because of their high sensitivity and selectivity, good portability, relatively low cost, rapid response, etc. For instance, the activity of protein kinases can be conveniently detected with ferrocenes (Fc) or Ag^{+} ions as the electro-active probes, which were either labeled to the substrate peptides in the presence of adenosine 5'-[γ-Fc] triphosphate (Fc-ATP) as the co-

* Corresponding author.
** Corresponding author.
*** Corresponding author.
E-mail addresses: j.kong@njust.edu.cn (J. Kong), zhangxueji@ustb.edu.cn (X. Zhang), ccywzhang@gzhu.edu.cn (Y. Zhang).

https://doi.org/10.1016/j.talanta.2019.120173
Received 15 May 2019; Received in revised form 8 July 2019; Accepted 24 July 2019
Available online 30 July 2019
0039-9140/

substrate [21] or were electrostatically adsorbed to the phosphorylated sites after the phosphorylation of the substrate peptides by protein kinases [22]. These methods are straightforward and easy in operation, but are suffered from finite sensitivity, since each phosphorylated substrate peptide can be labeled with only one electro-active probe. To substantially improve the sensitivity, several amplification strategies have been reported to date. For instance, the ssDNA-assembled AuNPs blocks (ssDNA-AuNPs) have been taken advantage of as carriers to enrich numerous $[Ru(NH_3)_6]^{3+}$ probes via the electrostatic adsorption for the electrochemical detection of protein kinase activity [23]. In addition, a phi29 DNA polymerase-mediated rolling circle amplification (RCA)-based electrochemical biosensor has been presented for the detection of protein kinase activity, since the RCA products (relatively long ssDNA fragments) can supply hundreds of sites for the electrostatic adsorption of $[Ru(NH_3)_6]^{3+}$ probes [24]. Although the electrochemical signals can be substantially amplified, the use of ssDNA-AuNPs as the amplifiers requires the synthesis and especially the tedious surface modification of AuNPs with the negatively charged ssDNA fragments, while the latter strategy necessitates the use of two short ssDNA fragments as the primer probe and the padlock probe and the phi29 DNA polymerase for RCA reaction and thus are suffered from high detection cost. So, it is still challenging to develop an easy-in-operation and low-cost electrochemical biosensor for the highly sensitive detection of protein kinase activity.

By virtue of their easy-in-operation, high efficiency, and low cost, the controlled/"living" radical polymerization (CLRP) techniques have recently been emerged as a novel class of amplification strategies in the highly sensitive detection of nucleic acids, antigens, and other clinically-related biological molecules [25–29], in which the surface-initiated grafting of long polymer chains based on the CLRP techniques, such as reversible addition-fragmentation chain transfer (RAFT) polymerization and atom transfer radical polymerization (ATRP), allows a drastic amplification of the detection signal. Amongst, the RAFT polymerization is rooted in the reversible transfer of the thiocarbonylthio group, S═C(Z)S−, of the RAFT agents such as dithiobenzoates between the propagating radicals and the dormant chains [30,31]. Since it is applicable to the polymerization of various vinyl monomers such as (meth)acrylates and even acrylic acid (AAc) in various solvent systems (e.g., bulk, solution, emulsion, and dispersion) and can be applied to the synthesis of polymers with specific molecular weight, relatively low dispersivity, and high end-group fidelity, RAFT polymerization has been widely investigated in the polymer synthesis and surface functionalization [31–33]. Thus far, as few as three reports concerning the application of the RAFT polymerization as a novel amplification strategy for DNA biosensing have also been demonstrated [25,27,34]. Although it is easy in operation, highly efficient, and low cost, the RAFT polymerization-based strategy has never been exemplified for the electrochemical detection of protein kinase activity.

In this work, a peptide-based electrochemical biosensor has been presented for the first time for the ultrasensitive detection of protein kinase activity by using the RAFT polymerization technique as an amplification strategy. Specifically, the substrate peptides were first tethered to a gold electrode surface via the thiol terminals and were used for the recognition of target protein kinase molecules. The substrate peptides did not contain any free carboxyl groups. Only after the site-specific phosphorylation of the substrate peptides by protein kinases, such that, the carboxyl-containing RAFT agents can then be labeled to the substrate peptides via the robust zirconium(IV) linkers [26]. In the presence of ferrocenylmethyl methacrylates (FcMMAs) as the monomers, the grafting of ferrocenyl polymer chains from the phosphorylated sites caused by the subsequent RAFT polymerization can efficiently recruit a great number of electro-active Fc probes to each phosphorylated site, leading to a drastic amplification of the electrochemical signal. Amplified by the RAFT polymerization, the peptide-based electrochemical biosensor allowed the ultrasensitive detection of protein kinase activity, without the need for complex operations or costly chemicals. With PKA as the target, for instance, the detection limit was as low as 1.05 mU mL^{-1}. Moreover, the peptide-based biosensor can selectively differentiate the target from other interferents and can be used for the screening of potential small-molecule inhibitors as well as the detection of protein kinase activity in complex cell lysates. Therefore, the peptide-based biosensor shows great promise as a universal tool for protein kinase activity detection and inhibitor screening.

2. Material and methods

2.1. Materials and reagents

Substrate peptides LRRASLGGGGC with the terminal carboxyl groups being blocked by amidation were provided by Ketai Biotechnology Co., Ltd. (Shanghai, China). PKA (0.4 U μg^{-1}, from bovine heart), thrombin (Tb), hemoglobin (Hb), human serum albumin (HSA), alkaline phosphatase (ALP), mercaptohexanol (MCH), cAMP, FcMMA, and ATP were supplied by Sigma-Aldrich (St. Louis). Human PKA ELISA kit, forskolin, Dulbecco's modified Eagle medium (DMEM, high glucose), 3-isobutyl-1-methylxanthine (IBMX), 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid (CPAD), casein kinase II (CK2), and fetal bovine serum (FBS) were offered by Jonln Industrial Co., Ltd. (Shanghai, China), Alomone Labs (Jerusalem, Israel), GE Healthcare Life Sciences (Logan, Utah), Yuanye Biotechnology Co., Ltd. (Shanghai, China), Aladdin Bio-Chem Technology Co., Ltd. (Shanghai, China), New England Biolabs Ltd. (Beverly, MA), and YiJi Industrial Co., Ltd. (Shanghai, China), respectively. KPF_6, $ZrOCl_2$, $LiClO_4$, 2,2′-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (AIBI), *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H-89), and 2-(*N*-morpholino)ethanesulfonic acid monohydrate (MES) were supplied by J&K Scientific Ltd. (Shanghai, China). Absolute ethanol (EtOH) and other analytically pure reagents were supplied by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All chemicals were used as supplied. Milli-Q water with a resistivity not lower than 18.25 MΩ cm was used throughout the work.

PKA solutions were prepared with 10 mM MES (pH 6.5), containing 10 mM $MgCl_2$, 0.6 mM cAMP, and 0.2 mM ATP. The solution for RAFT polymerization was a mixture of FcMMA (0.1 mM) and AIBI, and was freshly prepared with 15% DMF. The solutions of substrate peptide, MCH, Zr^{4+}, and CPAD were prepared with 20 mM MES (pH 6), 60% EtOH, 20% EtOH, and 20% EtOH, respectively.

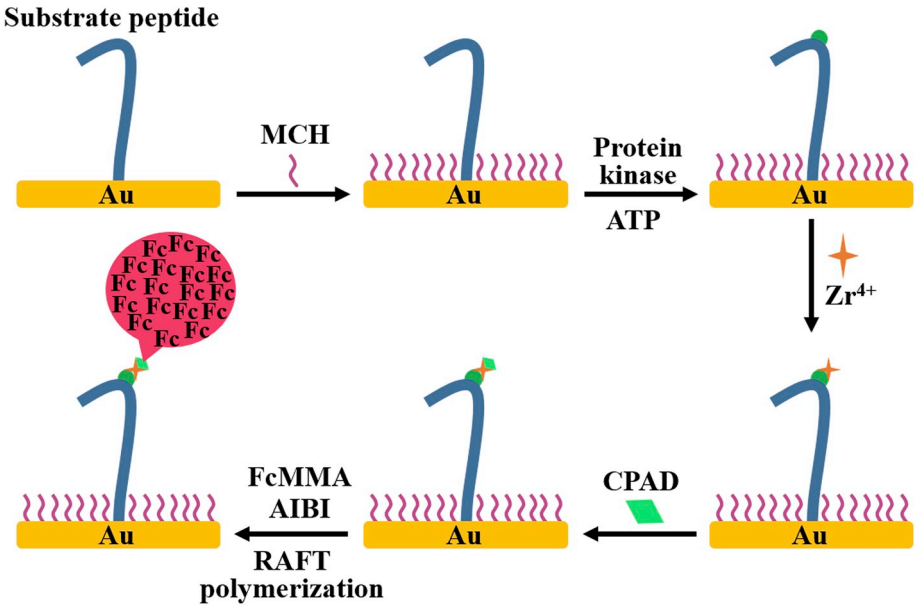
2.2. Apparatus

For details, please see the Supplementary Material.

2.3. Electrochemical detection of protein kinase activity

Prior to each use, gold electrode surface was renewed according to the following procedures. First, it was polished to a mirrorlike finish with 0.05 μm alumina slurry, sonicated in EtOH and Milli-Q water, and soaked in a 1:3 (v/v) mixture of H_2O_2 (30%) and H_2SO_4 (98%) for 10 min (*Caution! Piranha solution is highly corrosive!*) [26]. Next, it was rinsed with Milli-Q water and scanned in 0.5 M H_2SO_4 solution by CV, with a scan rate of 0.1 V s^{-1} and a potential range of −0.3–1.5 V, until a reproducible curve was obtained [16]. Last, it was soaked in $NaBH_4$ solution for 15 min [34], sonicated in Milli-Q water, and dried with N_2.

After the addition of 5 μL of 1 μM substrate peptide solution, the electrode was incubated for 1 h at 37 °C, and adequately rinsed with 20 mM MES (pH 6). Further, the electrode surface was blocked with MCH solution (2 mM) for 0.5 h, and rinsed with EtOH and Milli-Q water. Onto the electrode surface, 20 μL of PKA solution was pipetted, followed by another incubation for 1 h at 37 °C and an adequate rinse with 10 mM MES (pH 6.5). Then, it was soaked in Zr^{4+} linker solution (2 mM) for 0.5 h at 37 °C, and adequately rinsed with Milli-Q water.



Scheme 1. Schematic illustration of the design principle of the peptide-based electrochemical biosensor.

After soaking in CPAD solution (0.5 mM) for 0.5 h at 37 °C, it was rinsed with EtOH and Milli-Q water.

The electrode surface was further soaked in the RAFT polymerization solution for 1.5 h at 47 °C (the 10 h half-life decomposition temperature of AIBI is 44 °C [31]). After rinsing with DMF and Milli-Q water to remove any nonspecifically adsorbed species, the as-modified electrode was ready for the SWV test in 0.5 M $LiClO_4$ solution [26], with an increase potential of 4 mV, a potential amplitude of 25 mV, a frequency of 15 Hz, and a quiet time of 60 s.

3. Results and discussion

3.1. Design principle of the peptide-based electrochemical biosensor

Scheme 1 depicted the design principle of the peptide-based biosensor. First, the Cys-terminated substrate peptides were tethered to a gold electrode surface through the well-established gold-sulfur self-assembly chemistry. When PKA was used as the target, the substrate peptide contained a Kempeptide fragment LRRASLG, which has been known to be an ideal substrate (Michaelis constant (K_m) = 16 μM) for the catalytic subunit of PKA [35]. After the site-specific phosphorylation of the substrate peptides by protein kinases in the presence of ATP as the co-substrate, CPADs were labeled to the phosphorylated sites via the Zr^{4+} linkers and were served as the RAFT agents for the subsequent polymerization, which is initiated upon thermal decomposition of free radical initiators. Herein, AIBI was used as the free radical initiator, in view of its good water solubility and especially the relatively low decomposition temperature [34]. Using FcMMAs as the monomers, the polymerization-mediated grafting of ferrocenyl polymer chains can recruit a great number of Fc probes to the electrode surface (the mechanism has been detailed in ref. 34), giving rise to a drastic amplification of the electrochemical signal. As a result, protein kinase activity can be detected with an ultrahigh sensitivity, without the need for any complex operations or costly chemicals.

3.2. Characterizations

First of all, we verified the feasibility of the peptide-based biosensor. For comparison, the SWV curves of the differently modified electrodes were shown in Fig. 1A. For the as-modified electrode (curve "a"), a strong peak current (I_p) resulting from the one-electron oxidation of the Fc probes can be observed at ~0.3 V [26]. As can be observed from the scan rate-dependent CV curves (inset in Fig. 1B), there were good linear correlations between the redox currents and the scan rate. Thus, the observed redox behavior of the Fc probes was a surface-controlled process, indicating that they were confined and thus covalently tethered to the electrode surface [26]. As shown in the SEM image (Fig. 1C), after the polymerization, a high density of polymer dots were stacked on the electrode surface [34], signifying the high efficiency of the RAFT polymerization. The elemental compositions of the as-modified electrode were analyzed by energy-dispersive X-ray spectroscopy (EDS). The trace of P element (Fig. S1A) represented the successful phosphorylation of the substrate peptides by protein kinases. Particularly, the trace of Fe element (Fig. S1B), which was characteristic of the Fc probes, demonstrated that the dots were the ferrocenyl polymers [34]. As was also shown Fig. 1A, when the electrode surface was modified without the addition of substrate peptides (curve "b"), protein kinases (curve "c"), Zr^{4+} linkers (curve "d"), RAFT agents (curve "e"), free radical initiators (curve "f"), or FcMMA monomers (curve "g"), almost no peak current can be observed. The above results indicated that the peptide-based biosensor is quite feasible and reliable.

Further, EIS was applied to characterize the modifications of the electrode surface. The Nyquist plots were presented in Fig. 1D. As can be seen, the bare gold electrode showed a small charge-transfer resistance (R_{ct}) (~0.14 kΩ, plot "a"), indicating the fast charge-transfer kinetics. The tethering of substrate peptides and the further blocking of the residual sites with MCH raised the R_{ct} to ~0.48 kΩ (plot "b") and ~1.10 kΩ (plot "c"), respectively, and these were caused by the steric hindrance from the inert monolayer(s) [34]. After the phosphorylation of the substrate peptides, the R_{ct} increased to ~1.40 kΩ (plot "d"), because the negatively charged phosphate groups were repellent to the interfacial charge transfer [34]. The chelation of Zr^{4+} linkers to the phosphate groups raised the R_{ct} to ~1.70 kΩ (plot "e"). This observation can be ascribed to the electrostatic adsorption of $[Fe(CN)_6]^{3-/4-}$ to the chelated Zr^{4+} linkers, because the negatively charged $[Fe(CN)_6]^{3-/4-}$ can interfere with the charge transfer [26]. After the labeling of CPADs, the R_{ct} increased to ~1.93 kΩ (plot "f"), because $[Fe(CN)_6]^{3-/4-}$ were expelled from the electrode surface due to the increase of surface hydrophobicity [34]. After the polymerization, the R_{ct} decreased to ~0.71 kΩ (plot "g"), since the densely-stacked electroactive polymer chains can greatly facilitate the charge transfer [34]. The above results confirmed the successful modification of the

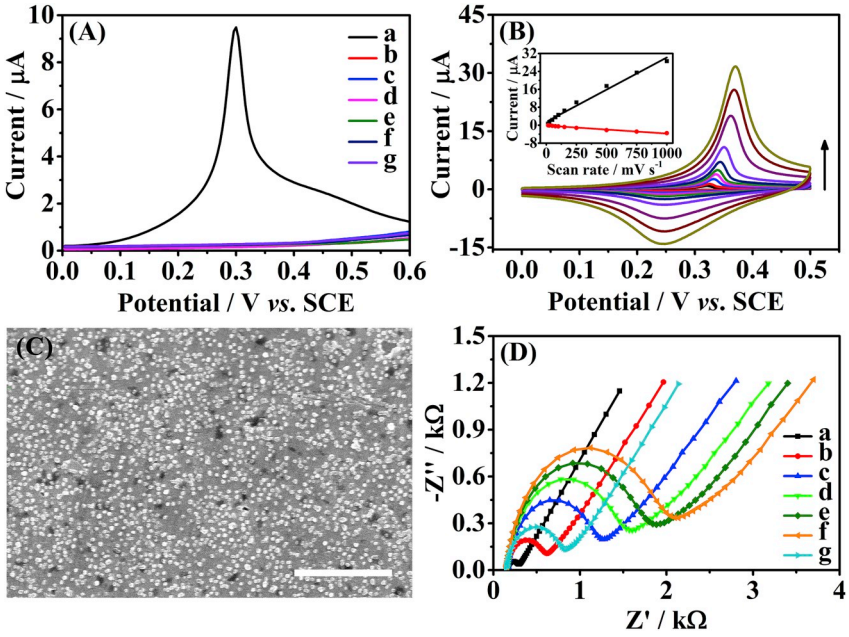


Fig. 1. (A) SWV curves of the as-modified electrode (a) and of those modified without substrate peptides (b), protein kinases (c), Zr^{4+} linkers (d), RAFT agents (e), free radical initiators (f), or FcMMA monomers (g). (B) Scan rate-dependent CV curves (from 15 to 1000 mV s^{-1}). Inset depicts the dependencies of redox peak currents on scan rate. The supporting electrolyte was 0.5 M $LiClO_4$. (C) SEM image of the as-modified electrode. The scale bar was 2 μm. (D) Nyquist plots of the bare gold electrode (a) and of those obtained after the modification of substrate peptides (b), MCHs (c), phosphorylation (d), Zr^{4+} linkers (e), CPADs (f), and ferrocenyl polymers (g). The activity of PKA was 140 mU mL^{-1}, the concentration of AIBI was 30 μM.

electrode surface.

3.3. Optimization of radical initiator concentration

In the polymerization, AIBI was used as the free radical initiator and it can be thermally decomposed to produce the initiating radicals upon cleavage of the C–N bonds [34]. Theoretically, a higher initiating radical concentration will result in a higher polymerization rate. However, this may deteriorate the livingness of the polymerization process, likely due to the radical-radical termination effect [31]. Hence, the concentration of radical initiator should be optimized. As can be seen from Fig. S2, with the increase of AIBI concentration from 15 μM to 30 μM, the peak current at ~0.3 V increased obviously; after that, it decreased significantly with the further increase of AIBI concentration to 45 μM. So, the polymerization was carried out in the presence of 30 μM AIBI.

3.4. Analytical performance of the peptide-based biosensor

Under the optimized conditions, the analytical performance of the peptide-based biosensor was investigated. As can be seen from the activity-dependent SWV curves (Fig. 2A), the peak current at ~0.3 V increased with the increase of protein kinase activity from 0 mU mL^{-1} to 140 mU mL^{-1}. That is, the activities of protein kinases can be detected in a "signal-on" mode, which is relatively tolerant to the false-positive results. As shown in Fig. 2B, the I_p value varied linearly along with the activity of protein kinases over the range from 0 mU mL^{-1} to 140 mU mL^{-1}, with a regression equation of I_p (μA) = 0.073 + 0.061 × *activity* (R^2 = 0.997), where I_p is the current value at ~0.3 V. Based on the $3\sigma_b$/slope criteria (where σ_b means the standard deviation of the blank control), the detection limit was estimated to be 1.05 mU mL^{-1}, which is much lower than those of the previously reported methods, as summarized in Table 1. Compared with the RCA-based electrochemical biosensor [24], for instance, the detection limit has been optimized by a factor of *ca.* 480 (1.05 mU mL^{-1} versus 500 mU mL^{-1}). The ultrahigh sensitivity can be ascribed to the RAFT polymerization, through which a great number of Fc probes can be recruited to the electrode surface, generating a relatively high electrochemical signal even in the presence of a very low level of protein kinase activity. The total cost of each assay of the peptide-based biosensor was calculated to be lower than 0.005 $; so, it is relatively low in detection cost.

3.5. Reproducibility, selectivity, and storage stability of the peptide-based biosensor

The reproducibility of the peptide-based biosensor was investigated with 140 mU mL^{-1} PKA, on the basis of the intra- and inter-batch assays that were independently accomplished using five identically modified electrodes. For the intra-batch assay, the variation coefficient of the I_p value was 4.5%, whereas for the inter-batch assay it was 5.2%. The results demonstrated that the peptide-based biosensor had acceptable reproducibility.

The selectivity of the peptide-based biosensor was evaluated using PKA, Tb, Hb, HSA, ALP, and CK2. The concentration of PKA was

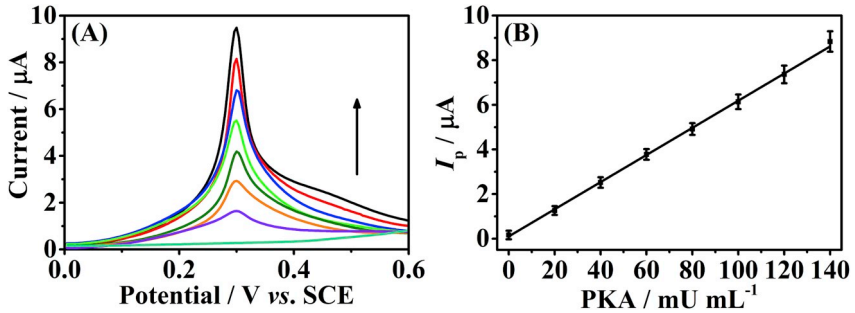


Fig. 2. SWV curves (A) and calibration plot (B) toward different PKA activities from 0 to 140 mU mL^{-1}. Error bars showed the standard deviations of five independent assays.

Table 1
Comparison of the analytical performance with those of other assays.

Method	Amplification strategy	Linear range[a] (U mL^{-1})	Detection limit (mU mL^{-1})	Mode	Ref.
PEC	Alkaline phosphatase	0.05–100	170	Signal-on	[9]
ECL	AuNPs	0.01–10	5	Signal-on	[10]
QCM	No	0.64–22.33	61	Signal-on	[11]
Fluorometric	No	Unavailable	500	Signal-off	[12]
Electrochemical	HCR[a]	0.004–400	1.5	Signal-on	[13]
Electrochemical	AuNPs/MWNTs[a]	100 − 1000	90	Signal-on	[18]
Electrochemical	ssDNA-AuNPs	0.03–40	30	Signal-on	[23]
Electrochemical	RCA	5–500	500	Signal-on	[24]
Electrochemical	RAFT polymerization	0–0.14	1.05	Signal-on	This work

[a] The normal range of extracellular PKA activity in human serum is 0–10.6 mU mL^{-1}; HCR: hybridization chain reaction; MWNTs: multi-walled carbon nanotubes.

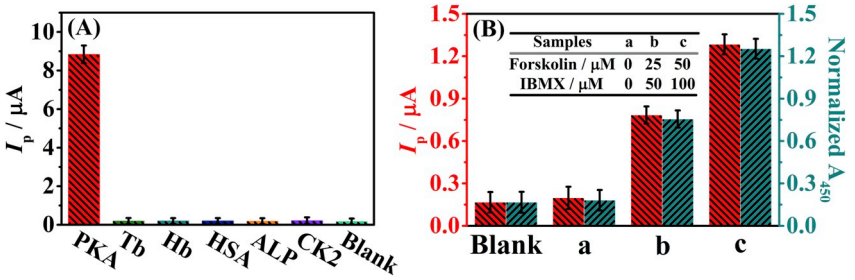


Fig. 3. (A) I_p values toward different enzymes or proteins. The concentration of PKA was 0.35 μg mL^{-1} (140 mU mL^{-1}), whereas of others were 3.5 μg mL^{-1}. (B) Values of absorbance at 450 nm (A_{450}; the values have been normalized by setting the A_{450} value of the blank control equal to that of the I_p value of the blank control) and I_p values toward different concentrations of forskolin/IBMX.

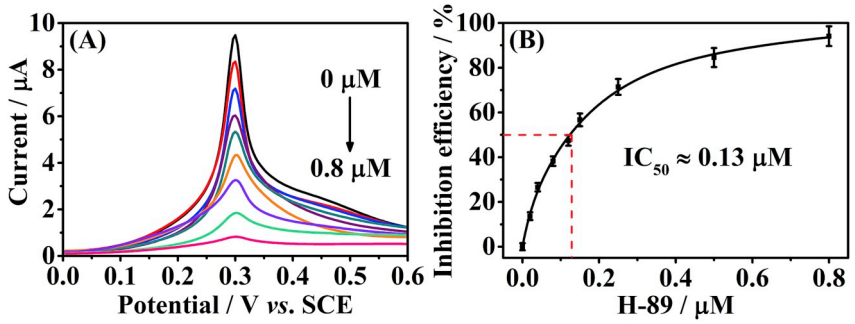


Fig. 4. (A) H-89 concentration-dependent SWV curves (from 0 to 0.8 μM). (B) Dependency of inhibition efficiency on H-89 concentration. The activity of PKA was 140 mU mL^{-1}.

0.35 μg mL^{-1}, whereas of others were 3.5 μg mL^{-1}. As shown in Fig. 3A, the addition of PKA led to a high I_p value, whereas the addition of others resulted in negligible changes in the I_p value relative to the blank control. Thus, the peptide-based biosensor can selectively differentiate the target from other interferents.

In addition, the storage stability of the as-modified electrodes was interrogated by a long-term storage test using five identically modified electrodes. The activity of PKA was 140 mU mL^{-1}. The electrodes were measured immediately after modification, and then were stored at 4 °C for two weeks before the secondary measurement. The negligible decrease in the I_p value indicated that the as-modified electrodes bore satisfactory storage stability.

3.6. Application to small-molecule inhibitor screening

To validate the application potential of the peptide-based biosensor in inhibitor screening, the detection of 140 mU mL^{-1} PKA was performed in the presence of different concentrations of inhibitors. As shown in Fig. 4A, with the increase of the concentration of H-89, a competitive inhibitor of PKA, the I_p value also decreased. The reason is that the inhibition of PKA activity by H-89 can slow down the phosphorylation of substrate peptides, which in turn resulted in the recruitment of fewer Fc probes. For PKA, the half-maximal inhibitory value (IC_{50}) of H-89 was around 0.13 μM (Fig. 4B), which was comparable with the previously reported value [16]. The results indicated that the peptide-based biosensor was applicable for inhibitor screening.

3.7. Analysis of complex cell lysates

The application potential of the peptide-based biosensor in complex biological samples was validated with HepG2 cell lysates (cell culture and lysate preparation were detailed in the Supplementary Material). As shown in Fig. 3B, for the unstimulated lysates, negligible change in the I_p value relative to the blank control (without cell lysates) can be observed, indicating the very low levels of intracellular PKA activity under normal physiological conditions [34]. For the stimulated lysates, a higher I_p value relative to the blank control is observed, with the value increasing with the dose of forskolin/IBMX. Furthermore, the results were comparable to those of the ELISA kit, showing that the peptide-based biosensor was applicable for the detection of protein kinase activity in complex cell lysates.

4. Conclusions

In summary, a “signal-on” peptide-based electrochemical biosensor has been presented for the ultrasensitive detection of protein kinase activity, using the RAFT polymerization technique as an operationally

simple and low-cost amplification strategy. Through the polymerization, a great number of electro-active Fc probes can be recruited to the electrode surface, generating a relatively high electrochemical signal even in the presence of a very low level of protein kinase activity. Amplified by the RAFT polymerization, the peptide-based electrochemical biosensor allows the detection of protein kinase activity with an ultrahigh sensitivity, without the need for any complex operations or costly chemicals. Furthermore, the peptide-based biosensor features a high selectivity even in the presence of tenfold amounts of other interferents and is highly applicable for the screening of potential small-molecule inhibitors and the detection of protein kinase activity in complex cell lysates. Therefore, it shows great promise as a universal tool for protein kinase activity assay and inhibitor screening.

Acknowledgments

This work was supported by the Project Funded by China Postdoctoral Science Foundation [grant number 2018M643040], and the National Natural Science Foundation of China [grant numbers 21527806, 21874031, 21575066, 21727815].

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2019.120173.

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