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Highly Sensitive Homogeneous Electrochemiluminescence Biosensor for Alkaline Phosphatase Detection Based on Click Chemistry-Triggered Branched Hybridization Chain Reaction

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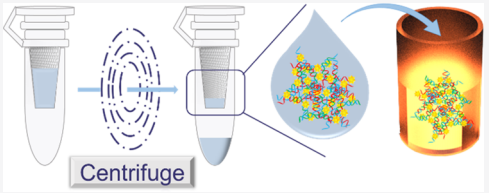
ABSTRACT: Alkaline phosphatase (ALP) has been used as a diagnostic index of clinical diseases since its expression level is closely related to many pathological processes. In this work, a highly sensitive electrochemiluminescence (ECL) method for the determination of ALP based on a click chemistry-induced branched hybridization chain reaction (BHCR) for signal amplification and ultrafiltration technology for the separation of homogeneous amplification products is introduced. ALP can release copper ions from a Cu^{2+}/PPi complex by hydrolyzing pyrophosphoric acid, which initiates click chemistry in the system. A BHCR amplification is triggered afterward by the long single-stranded DNA (ssDNA) generated by click chemistry, resulting in a three-dimensional double-stranded DNA (dsDNA) with a large molecular weight. Based on the characteristic that $Ru(phen)_3^{2+}$ can stably insert into the groove of dsDNA, a large amount of $Ru(phen)_3^{2+}$ is retained together with the amplified product after ultrafiltration, and therefore a significantly enhanced ECL signal can be obtained. The test results show that this method can be used for the quantitative determination of ALP ranging from 0.002 to 50 U/L, with a detection limit of 0.7 mU/L. This method has also been confirmed to have good selectivity and anti-interference, and the results of the analysis of the ALP content in the diluted serum samples are satisfactory, showing great application potential in clinical diagnosis.



Alkaline phosphatase (ALP) is a membrane metalloenzyme in biological tissues that hydrolyzes phosphate monoesters to achieve the dephosphorylation process of substrates.[1−3] Studies have shown that the occurrence of bone cell carcinoma, prostate cancer, liver injury, renal function deterioration, and other diseases is often accompanied by the abnormal expression of ALP.[4−8] In view of the close correlation between ALP and multiple pathological processes, the content of ALP in serum or whole blood has been clinically used as an important indicator for the diagnosis and prognosis of hepatobiliary and skeletal diseases.[9,10] Therefore, accurate detection of ALP activity can provide more reliable evidence for the early diagnosis of clinical diseases, which is also a major challenge for current research. Many analytical techniques based on electrochemistry, fluorescence, colorimetry, etc. had been adopted to develop ALP biosensors.[11−13] As an analytical technique combining electrochemistry and chemiluminescence, the electrochemiluminescence (ECL) detection technique has the advantages of simple equipment, high sensitivity, and easy operation.[14,15] This technology has been applied to detect ALP early. For example, Qi and co-workers used p-nitrophenyl phosphate as the hydrolysis substrate of ALP and constructed an ECL immunoassay method responding to ALP based on the characteristics of ECL resonance energy transfer between p-nitrophenol and luminol.[16] Wang and his co-researchers prepared a perovskite quantum dot with high ECL efficiency and applied it to detect ALP with a detection limit of 0.714 mU/L.[17] Yang and co-workers proposed an ultrasensitive ECL biosensor based on a closed bipolar electrode and applied it to the determination of ALP in a single Hep G2 cell.[18]

The Cu^{+}-catalyzed alkyne−azide cycloaddition (CuAAC) reaction has the characteristics of rapid reaction, high efficiency, and mild conditions, which make it suitable for the detection of biological samples.[19] Utilizing the hydrolysis characteristics of ALP to its hydrolyzed substrate can realize the reduction process of Cu^{2+} to Cu^{+}, which in turn triggers the click chemistry reaction. This mechanism had been applied to develop a sensitive biosensor. For example, Jiang et al. designed a naked-eye readable plasma nanosensor using ALP-triggered agglomeration of gold nanoparticles and demonstrated its potential in immunoassay applications.[20] Applying the ALP-labeled antibody to the immune sandwich structure and employing the same mechanism, Wu and co-workers established a magnetic resonance sensing-based immunoassay

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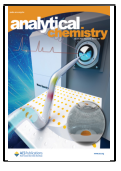

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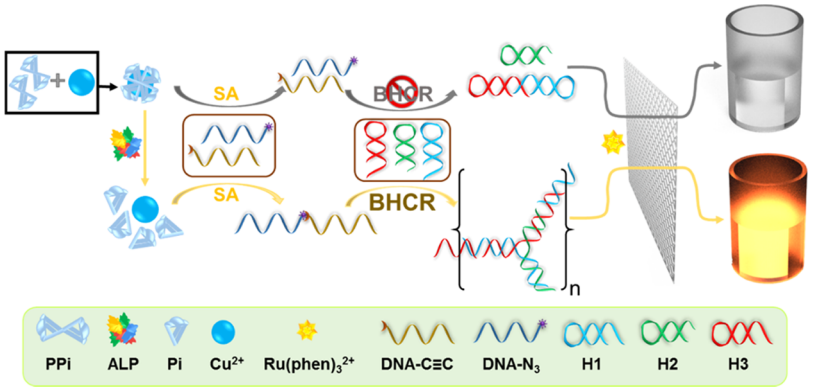
Scheme 1. Schematic Illustration of the Proposed ECL Biosensor for ALP Detection Based on Click Chemistry-Induced BHCR Amplification



platform for the detection of small molecules.[21] Early studies indicated that the cyclization reaction between the specially designed 5′-azide and 3′-alkyne tagged padlock probes through CuAAC can produce a relatively long single-stranded DNA (ssDNA), which nearly has the same capability as a normal ssDNA and has been used to trigger hyperbranched rolling circle amplification (HRCA) and produce large amounts of double-stranded DNAs (dsDNAs) with high efficiency.[22] Since the addition of SYBR Green I in a dsDNA solution can produce enhanced fluorescence, this had been applied to develop a highly sensitive fluorescence biosensor for inorganic pyrophosphatase detection.[23]

As an ECL signal molecule, $Ru(phen)_3^{2+}$ can be embedded into dsDNA with the dual effects of covalent binding and electrostatic affinity.[24−26] Combined with some nucleic acid amplification technologies such as hybridization chain reaction (HCR) and HRCA, which can produce a large number of dsDNA, many ECL sensors that rely on $Ru(phen)_3^{2+}$ for signal output have been constructed.[27−29] Most of these sensors immobilized the DNA probe on the surface of the working electrode. Although high sensitivity can be obtained, electrode processing is cumbersome and the efficiency of nucleic acid hybridization on the electrode surface is also limited. An effective way to solve this problem is to design a homogeneous amplification system and then purify and enrich the amplification products using ultrafiltration technology according to the molecular weight difference between the amplification materials and the products. Branched hybridization chain reaction (BHCR) is a derivative of HCR, which is different from HCR in that a third kind of hairpin probe is introduced and three-dimensional dsDNA products can be obtained.[30,31] BHCR retains the nonenzymatic and isothermal amplification characteristics of HCR. Its unique multiple reaction orientations destined that its reaction kinetics is greatly accelerated and the amplification efficiency is much higher than that of HCR. The products of BHCR have the characteristics of large molecular weight and three-dimensional structure, which make it a good match for the separation of products by ultrafiltration technology. The homogeneous BHCR strategy of enrichment and purification of amplification products by ultrafiltration has not been combined with ECL technology for target analysis.

In this study, an ECL biosensor for ALP was developed, which combined the high selectivity of CuAAC for the target-specific response and the high amplification efficiency of BHCR amplification technology and used ultrafiltration technology to separate the amplification products with a large molecular weight to eliminate the cumbersome electrode modification procedure. The principle of the proposed sensing strategy for ALP activity detection is shown in Scheme 1. Pyrophosphate ion (PPi) chelates with Cu^{2+} to form a Cu^{2+}/PPi complex. As ALP can hydrolyze PPi to orthophosphate (Pi), it releases the Cu^{2+} from the stable coordination complex formed by Cu^{2+} and PPi. Subsequently, Cu^{2+} is reduced to Cu^{+} by sodium ascorbate (SA) and catalyzes the click chemistry reaction between the terminal alkyne group of DNA-C≡C and the azide group of $DNA\text{-}N_3$, so that the two short ssDNAs are linked into a longer ssDNA (L-ssDNA); this was specifically designed to be used as a trigger chain to induce BHCR amplification in the system, resulting in three-dimensional amplification products of large molecular weight. $Ru(phen)_3^{2+}$ is embedded in the dsDNA groove of the amplification product and is retained in the ultrafiltration tube along with the amplification product. Therefore, a strong ECL signal can be obtained by detecting the retentate. Conversely, without the participation of ALP, Cu^{2+} is confined to the Cu^{2+}/PPi complex and would not be reduced, thus hindering click chemistry and BHCR formation in the system. In this case, $Ru(phen)_3^{2+}$ and the amplification material are filtered out during the ultrafiltration process due to their small molecular weight, so the ECL signals of retentate are relatively weak. The measured ECL signal strength is positively correlated with ALP activity, and ALP can be quantified by measuring ECL intensity. The ECL biosensor for ALP detection in this work is simple in operation and has good repeatability, which can realize the ultra-high sensitivity detection of ALP. Moreover, the designed ALP-sensing strategy shows good quantitative performance for the determination of ALP activity in serum samples and has potential applications in clinical diagnosis.

EXPERIMENTAL SECTION

Materials. Alkaline phosphatase (ALP, EC 3.1.3.1), dichlorotris(1,10-phenanthroline)ruthenium(II) hydrate ($Ru(phen)_3^{2+}$), and tripropylamine (TPA) were purchased from Sigma-Aldrich (Shanghai, China). Pyrophosphate ion (PPi) was provided by Beilian Fine Chemicals Co., Ltd. (Tianjin, China). Sodium ascorbate (SA) and copper sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$) were purchased from Alfa Aesar China Co., Ltd. (Tianjin, China). Thrombin, human serum albumin (HSA), trypsin, and bovine serum albumin (BSA) were provided by Macklin Biochemical Co., Ltd. (Shanghai, China). Tris-borate-EDTA (5× TBE) and all oligonucleotides used in

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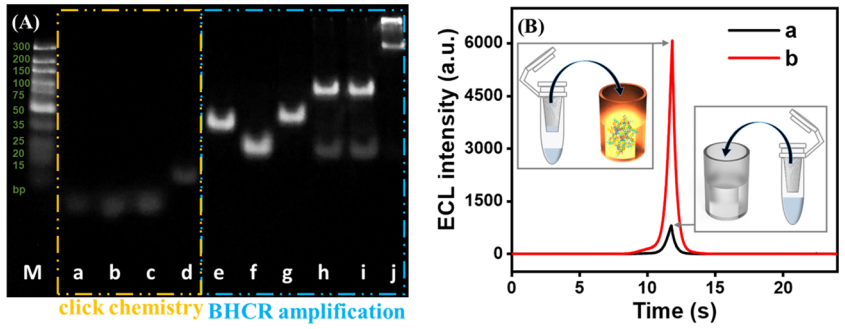

Figure 1. (A) Polyacrylamide gel electrophoresis identification of different conditions (lane M: marker, lane a: DNA-N_3, lane b: DNA-C≡C, lane c: DNA-N_3 + DNA-C≡C + Cu^{2+}/PPi, lane d: DNA-N_3 + DNA-C≡C + Cu^{2+}/PPi + ALP, lane e: H1, lane f: H2, lane g: H3, lane h: H1 + H2 + H3, lane i: DNA-N_3 + DNA-C≡C + Cu^{2+}/PPi + H1 + H2 + H3, and lane j: DNA-N_3 + DNA-C≡C + Cu^{2+}/PPi + ALP + H1 + H2 + H3). (B) ECL spectra of the proposed sensing system in the (a) absence and (b) presence of 5 U/L ALP.

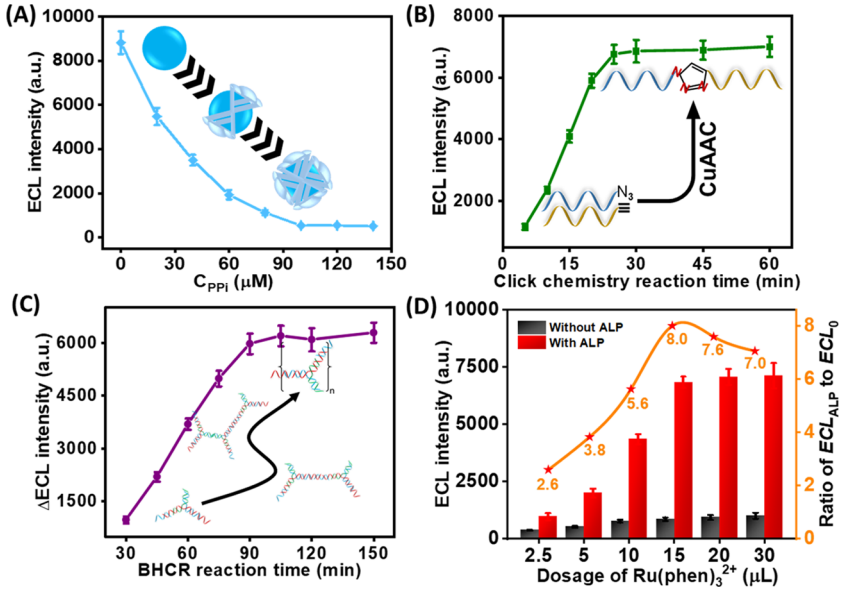

Figure 2. Optimization of some experimental parameters: (A) effect of PPi concentration on the background ECL intensity; (B) effect of the time of click chemistry reaction on ECL response; (C) effect of the time of BHCR reaction on ECL response; and (D) effect of the dosage of $Ru(phen)_3^{2+}$ on ECL response and SNR. ALP (10 U/L) was used for the above optimization experiments. The illustrated error bars represent the standard deviation of three replicates.

this work were purchased from Sangon Inc. (Shanghai, China). The oligonucleotide sequences are as follows (DNA-N_3 oligonucleotide was functionalized with 3′-azide, and DNA-C≡C oligonucleotide was functionalized with 5′-alkyne.)

DNA-N_3: 5′-CTATGACTATCA-azide-3′

DNA-C≡C: 5′-alkyne-ACCCTGCCGCCG-3′

H1: 5′-CGGCGGCAGGGTTGATAGTCATAGTCCAATCACAACCTATGACTATCAACCCTGAACCACCACCAACCACCACC-3′

H2: 5′-TGATAGTCATAGGTTGTGATTGGAACCCTGCCGCCGTCCAATCACAACCTATGA-3′

H3: 5′-GTTGTGATTGGACGGCGGCAGGGTCTATGACTATCAACCCTGCCGCCGTCCAATGGTGGTGGTTGGTGGTGGTT-3′

Apparatus. The electrochemical workstation (CHI660D) was purchased from Shanghai Chenhua Instruments Co., Ltd. (Shanghai, China). The BPCL ultraweak luminescence analyzer was obtained from the Institute of Biophysics, Chinese Academy of Science (Beijing, China). The electrophoresis analyzer (DYY-8C) was purchased from Beijing Liuyi Biological Technology Co., Ltd. (Beijing, China). The gel electrophoresis imager (JS-2012) was obtained from Shanghai Peiqing Technology Co., Ltd. (Shanghai, China). The glassy carbon electrode (3 mm in diameter), platinum wire electrode, and Ag/AgCl electrode were purchased from Wuhan Gaoshiruilian Technology Co., Ltd. (Hubei, China) and used as the working electrode, counter electrode, and reference electrode, respectively.

Procedures of ALP Detection. Cu^{2+} (40 μM) and PPi (100 μM) were mixed at room temperature for 20 min to form a stable Cu^{2+}/PPi coordination complex ($Cu(P_2O_7)_2^{6-}$) first. By adding a certain amount of ALP to the mixture and reacting at 37 °C for 45 min, Cu^{2+}, which was previously confined in Cu^{2+}/PPi, returned to a free state due to the hydrolysis of PPi by ALP. Thereafter, DNA-C≡C (2 μM), DNA-N_3 (2 μM), and SA (10 μM) were added to the solution, mixed uniformly, and incubated at 37 °C for 25 min, so that DNA-C≡C and DNA-N_3 formed an L-ssDNA through click chemistry. Subsequently, three material hairpin probes (H1, H2, and H3, with a concentration of 20 μM each) for BHCR amplification were added to the above reaction solution, and the amplified products were obtained after 90 min at 37 °C.

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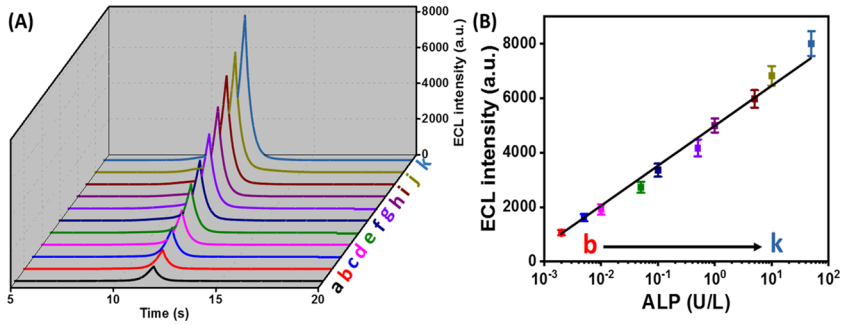

Figure 3. (A) ECL responses for different ALP concentrations (a–k): 0, 0.002, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, and 50 U/L, respectively. (B) Linear relationship between ECL intensity and the logarithm of the ALP concentration. The illustrated error bars represent the standard deviation of three replicates.

After that, 15 μL of $Ru(phen)_3^{2+}$ (10 μM) was added to the system and incubated for 30 min to allow $Ru(phen)_3^{2+}$ to be fully embedded in the dsDNA. After the above steps, the ultrafiltration centrifuge tube with a molecular weight cutoff of 100 kD was used to enrich the large molecular weight amplification products produced, and the enriched retentate was used for further ECL detection.

The retentate obtained in the foregoing process was mixed with 20 mM TPA in 1 mL of phosphate-buffered saline (PBS) buffer, and the ECL signal of the mixture was recorded. Each sample was measured three times and the average value was used for quantitative analysis. The ECL signals were recorded in the voltage range of 0.4–1.6 V, and the other parameters set during the detection were listed as follows: photomultiplier tube voltage = −800 V, scan rate = 100 mV/s.

Polyacrylamide Gel Electrophoresis Analysis. A 12% polyacrylamide gel was used for electrophoresis analysis of the materials and the products of each step. The electrophoresis was performed in 0.5× TBE buffer, and the working voltage was set to 80 V. The results of electrophoresis were photographed using a JS-2012 gel imager.

RESULTS AND DISCUSSION

Feasibility of the Proposed Biosensor for ALP. Gel electrophoresis is an intuitive and convincing method to verify whether the click chemistry reaction has occurred in the system and whether the designed oligonucleotide chain has successfully connected by click chemistry and be used to trigger a BHCR amplification. As shown in Figure 1A, lane a and lane b represent DNA-N_3 and DNA-C≡C, respectively. Compared with lane c (without ALP), a newly generated band with a larger molecular weight could be observed in lane d after adding ALP, indicating that the addition of ALP can release Cu^{2+} from the complex and the Cu^+ generated by reduction can induce click chemistry to connect DNA-N_3 and DNA-C≡C to L-ssDNA. When no target ALP is present, the band position of the system (lane i) is similar to that of the mixture containing the three hairpin materials of BHCR only (lane h), indicating that no obvious amplification reaction occurred in the system. In the presence of ALP, bright bands appeared at the top of lane j, indicating that the L-ssDNA as a trigger chain successfully induced the BHCR amplification in the system and produced high molecular weight amplified products. The results of polyacrylamide gel electrophoresis confirmed that ALP could be used to regulate click chemistry and BHCR amplification through the designed method.

Table 1. Comparison Between Other Strategies and the Developed ECL Biosensor for ALP Detection

detection method	dynamic range (U/L)	detection limit (U/L)	refs
photoelectrochemical method	0.04–400	0.022	32
fluorescent method	0.1–20	0.2	33
colorimetric method	0.8–6	0.26	34
electrochemical method	20–1500	3	35
electrochemical method	1–1000	0.4	36
ECL method	5–50	0.8	16
ECL method	0.002–50	0.0007	this work

The feasibility of the proposed ECL biosensor was explored by adding $Ru(phen)_3^{2+}$ to the amplified solution and performing ECL detection after ultrafiltration and centrifugal separation (Figure 1B). Without the target, the maximum molecular weight of the substance in the system is about 45 kD (the hybrid complex of H1 and H3), and the molecular weight of the primer–probe and $Ru(phen)_3^{2+}$ is relatively small and most of them are filtered out after ultrafiltration. The measured ECL signal of the retentate is very low (curve a). However, BHCR amplification occurred in the system when the target was present. The products of BHCR amplification are dsDNA with a three-dimensional structure and their relative molecular weight is very large (much greater than 100 kD). Most of the $Ru(phen)_3^{2+}$ embedded in the groove of dsDNA of the amplified products were retained in the ultrafiltration tube along with the amplification products during the centrifugation process. Hence, in this case, a strong ECL signal of the retentate can be measured (curve b).

Optimization of the Experimental Conditions. PPi can form a complex with Cu^{2+} and thus prevent the reduction of Cu^{2+} to Cu^+. Therefore, the concentration of PPi would affect the reaction efficiency of Cu^+-catalyzed click chemistry in the system, which in turn affects the BHCR amplification. Insufficient PPi will result in a high background signal, while excessive PPi will consume ALP and reduce the sensitivity of detection, so the concentration of PPi was first optimized in this experiment. As depicted in Figure 2A, the background ECL signal decreases with the increase of the PPi concentration and reaches the platform at 100 μM and tends to be stable, indicating that 100 μM PPi is sufficient to complex Cu^{2+} in the system fully. In addition, under the premise of low background signal, PPi at this concentration can maximize the detection sensitivity. The trigger chain of the

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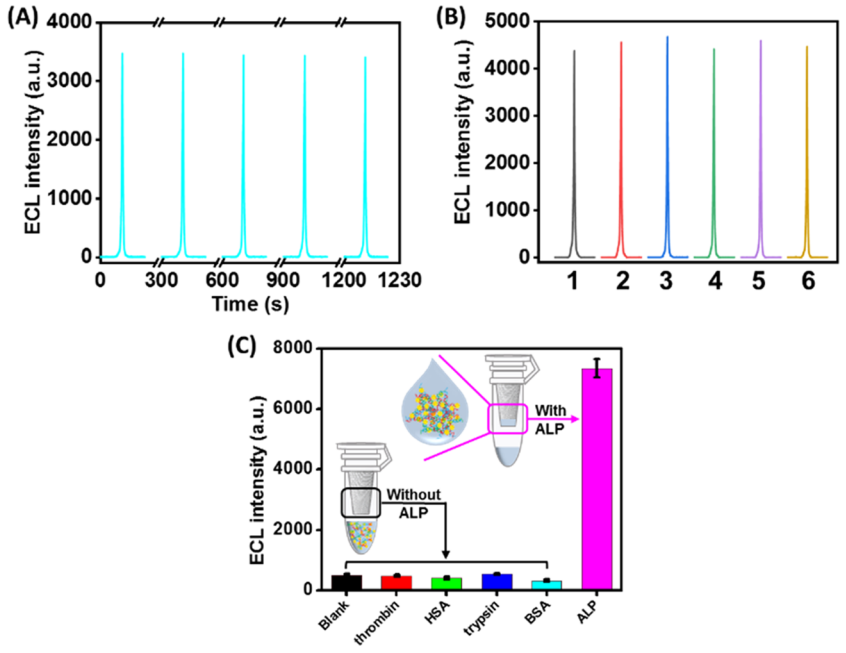

Figure 4. (A) ECL stability test of the proposed biosensor in the presence of 0.1 U/L ALP. (B) Reproducibility test of the proposed biosensor in the presence of 0.5 U/L ALP. (C) Selectivity test of the proposed biosensor in the presence of 10 U/L ALP and other analytes (50 μg/mL thrombin, 50 μg/mL HSA, 50 μg/mL trypsin, and 50 μg/mL BSA). The illustrated error bars represent the standard deviation of three replicates.

Table 2. Determination of the ALP Level in Diluted Human Serum Samples (n = 3)

sample no.	added (U/L)	found (U/L)	recovery rate (%)	RSD (%)
1		1.37		4.33
	1. 00	2.41	104	3.26
	10.0	11.5	101	3.84
2		0.68		3.79
	1. 00	1.65	97.0	4.81
	10.0	10.7	100	3.05
3		1.54		3.18
	1.00	2.59	105	3.64
	10.0	11.7	102	2.53

BHCR amplification is generated by the click chemistry of DNA-C≡C and DNA-N_3, and only sufficient click chemistry can make the amplification result of BHCR effectively feedback the response of the system to ALP. Therefore, the reaction time of click chemistry is also an important parameter that needs to be optimized. As shown in Figure 2B, the measured ECL intensity increased with the increase of the reaction time of click chemistry and approached the platform at 25 min. So, 25 min was selected for click chemistry in the subsequent experiments.

The amplification efficiency of BHCR is the key to the detection sensitivity of the proposed method. The reaction time of BHCR was also optimized so as to obtain the best amplification result in a short time. As shown in Figure 2C, when the reaction time increased from 0 to 90 min, the measured ΔECL intensity (ΔECL = ECL − ECL_0; ECL was the measured ECL intensity in the presence of the target, and ECL_0 was the ECL intensity when no target was present) increased rapidly, and even if the reaction time continued to increase, ΔECL intensity had no distinct change. Therefore, 90 min was chosen as the optimal BHCR amplification time.

To obtain a high signal-to-noise ratio (SNR), the effect of the dosage of $Ru(phen)_3^{2+}$ was also studied. As can be seen from Figure 2D, as the dosage of $Ru(phen)_3^{2+}$ increased, the background ECL intensity increased slightly. The reason is that the inner wall of the ultrafiltration tube has a certain adhesion and part of the $Ru(phen)_3^{2+}$ may adhere to the inner wall of the tube and get collected in the retentate, resulting in a background signal. In the presence of ALP, as the dosage of ruthenium increased, more and more $Ru(phen)_3^{2+}$ was embedded in the amplification product and was enriched in the retentate to output a signal. As a result, the measured ECL intensity also increased. When the dosage of ruthenium exceeded 15 μL, the ECL intensity hardly increased, indicating that the $Ru(phen)_3^{2+}$ embedded in the amplified product at this time was close to saturation, and the excess $Ru(phen)_3^{2+}$ was filtered out through the subsequent ultrafiltration process. The best signal resolution performance was determined by calculating the ratio of ECL_{ALP} to ECL_0 at different dosages of ruthenium. The result showed that the best signal resolution performance of the system was obtained when the dosage of $Ru(phen)_3^{2+}$ was 15 μL.

Performance of the Proposed System. Having confirmed these conditions, the ECL response of the proposed sensing system toward ALP with different activities was studied and is shown in Figure 3A. The result showed that as the concentration of ALP increased, the ECL intensity also increased. In addition, Figure 3B shows good linearity between ECL intensity and the logarithm of ALP concentration in the range of 0.002−50 U/L with a linear fitting equation of ECL = 1468 lg C_{ALP} + 4953 and a linear correlation coefficient (R) of 0.9954. The limit of detection (LOD) was estimated to be 0.7 mU/L (S/N = 3). Compared with the ALP detection method in the previous reference, although the sensor proposed in this study has no advantage in detection time, the detection sensitivity and dynamic range are greatly improved and complex materials are not required (see Table 1).

The ECL stability of the biosensor was analyzed by measuring once every 5 min in PBS buffer (containing 20

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mM TPA) with 100 mV/s as the scanning rate. As shown in Figure 4A, the peak ECL intensity is very stable in the five scans; there is no significant decrease, which shows that the biosensor has good stability.

It is worth noting that ultrafiltration technology was used in this study instead of the tedious electrode modification process in conventional ECL nucleic acid biosensor to achieve phase separation, thereby effectively eliminating the influence of electrode difference on the detection results, which not only simplifies the operation process but also ensures the reproducibility of the method. Six ultrafiltration tubes treated with NaOH solution were used to perform ultrafiltration and detection for the same sample (0.5 U/L ALP), thereby obtaining the reproducibility curve (as shown in Figure 4B). The relative standard deviation of ECL intensity was 2.54%, and the reproducibility was acceptable. These results confirm the superior stability and reproducibility of the designed ALP biosensor.

The selectivity of the proposed method was demonstrated by the study of several potential interferences including ATP, trypsin, HSA, thrombin, and BSA. As displayed in Figure 4C, this method showed superior specificity for ALP, and the signal of the system did not change significantly with the presence of related interfering proteins. This is mainly because only ALP can hydrolyze PPi and destroy the stability of the Cu^{2+}/PPi complex and therefore trigger the click chemistry reaction to induce BHCR amplification, resulting in an enhanced ECL signal.

Application of the Proposed Biosensor. The practicability of the proposed ECL sensor is the focus of the research. Inspired by the high sensitivity and selectivity of this method for ALP, we attempted to evaluate the ALP activity in human serum samples diluted 100-fold by the standard addition method. The results in Table 2 show that the method has strong anti-interference ability, can directly determine the ALP content in diluted serum samples, and the recoveries of ALP in different human serum samples are concentrated in 95.3−103.6%, with good accuracy. Hence, the proposed method is suitable for the quantitative determination of real samples.

CONCLUSIONS

In conclusion, based on the combination of click chemistry and BHCR technology, an ECL nucleic acid biosensor without electrode surface modification was developed for the determination of ALP activity. Compared with the current conventional ECL nucleic acid sensors, this strategy adopts the simple ultrafiltration technology, effectively solves the problem of cumbersome electrode processing operations of ECL nucleic acid biosensors, and successfully avoids the limitation of the steric hindrance to the amplification efficiency, which provides a new idea for the development of homogeneous ECL sensor. According to the test results, ALP in the range of 0.002−50 U/L can be detected quantitatively by this method, and the detection limit is as low as 0.7 mU/L. In addition, this method has been confirmed to be applicable in the determination of ALP in serum samples. In short, the sensor is easy to operate, with high sensitivity and strong anti-interference ability, and has a great application prospect in the clinical diagnosis of ALP quantitative determination.

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Notes

The authors declare no competing financial interest.

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