Rapid Communications

Highly Sensitive Alkaline Phosphatase Biosensor Based on Internal Filtration Effect between G-Quadruplex/*N*-methylmesoporphyrin IX and *p*-Nitrophenol

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In this work, an alkaline phosphatase (ALP) biosensor was established based on G-quadruplex/*N*-methylmesoporphyrin IX (G4/NMM) and *p*-nitrophenol (PNP). Because the absorption of PNP was close to the excitation wavelength of G4/NMM, PNP could reduce the fluorescence of G4/NMM. Meanwhile, PNP was the hydrolysis product of *p*-nitrophenylphosphate (PNPP) by ALP. Therefore, ALP could be detected. This ALP biosensor had a linear analytical range from 2.5 to 25 U/L a the detection limit of 0.81 U/L. Moreover, it showed a satisfactory selectivity and recovery rates.

Keywords Alkaline phosphatase, G-quadruplex, *p*-nitrophenol, inner filter effect, biosensor

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Alkaline phosphatase (ALP) with a dephosphorization function was widely found in human liver, bone, intestine, kidney, placenta and other tissues.[1] Because it plays important roles in signal transduction, cell growth and apoptosis, it has often been as a marker for specific diseases, such as bile-duct obstruction, abnormal liver function, and renal osteodystrophy.[2-4] Therefore, it was of great significance to detect the level of alkaline phosphatase in clinics.

So far, many methods have been developed for the determination of ALP, including electrochemical methods,[5-7] colorimetry methods,[8-10] and fluorescence methods.[11-13] Among them, fluorescence methods have attracted great attention due to their high sensitivity. Some fluorescence materials, such as nanoclusters,[14,15] organic fluorophores,[16,17] quantum dots[18-20] and carbon dots,[21,22] have been successfully used to monitor ALP. However, most of these methods were subject to some limitations, such as expensive reagents, a long synthesis time and complex synthesis routes. In addition, a commercial product 4-methylumbellferyl phosphate was developed to detect ALP, which was converted to fluorescent 4-methylumbellferone with 440 nm emission wavelength by ALP.[23] Unfortunately, many biological autofluorescences were at this wavelength, such as NAD(P)H (460 nm) and pterins (450 nm).[24,25] A long wavelength of greater than 600 nm was one effective method to avoid spontaneous fluorescence. Therefore, it was of great significance to explore a novel method for the detection of ALP.

G-Quadruplex (G4) was a special cage structure of DNA, which was formed by polyguanine.[26] Basically, four guanines could assemble into G-quartet by Hoogsteen-type base pairing. Then, two or more G-quartets could assemble into G4 by π-π stacking interactions.[27] Because porphyrin compounds had a planar aromatic ring structure, they could insert into G4 *via* π-π stacking interactions.[28] *N*-Methylmesoporphyrin IX (NMM) was a commercial asymmetric porphyrin. The fluorescence of NMM was very weak due to low solubility with 610 nm emission wavelength. When it bonded to G4, the fluorescence would increase dramatically. Thus, G4/NMM pair was often used as a label-free fluorescence reporter to design biosensor and avoid biological autofluorescence. Moreover, G4/NMM had been reported for the detection of ALP. For example, He *et al.* designed an ALP biosensor based on 5′-phosphorylated hairpin DNA and a λ exonuclease cleavage reaction in 2017.[29] Although this biosensor was facile and sensitive, it required additional enzymes and specific DNA. In 2020, another G4/NMM ALP biosensor was developed by the Kong group. In this biosensor, Cu^{2+} was used to quench the fluorescence of G4/NMM. Also pyrophosphate (the hydrolytic substance of ALP) can recover the fluorescence by binding with Cu^{2+}.[30] Although the sensitivity of this biosensor was satisfactory with a detection limit of 0.3 U/L, it needed an extra Cu^{2+} reaction step. Thus, a simpler G4/NMM biosensor was still needed.

p-Nitrophenylphosphate (PNPP) was a common substrate for measuring the ALP level. When it was hydrolyzed by ALP to become PNP, a characteristic absorption peak at 400 nm could be observed.[31] Accordingly, ALP could be detected by measuring the absorbance of PNP. However, in general, the absorbance method was less sensitive than the fluorescence method. Therefore, the conversion of the PNP absorbance signal into a fluorescence signal might increase the sensitivity. Coincidentally, the maximum excitation wavelength of G4/NMM was also 400 nm. Thus, PNP could quench the fluorescence of G4/NMM by an internal filtering effect (IFE).[32] Through PNPP/PNP mediation, ALP could be detected by G4/NMM as a label-free fluorescence reporter.

The principle of the designed biosensor is depicted in Scheme 1. Without ALP, the G4/NMM complex could emit intense fluorescence under the 400 nm excitation wavelength. When ALP was added, PNPP was hydrolyzed to PNP. Afterwards, because PNP had a strong absorption peak at 400 nm, the fluorescence of G4/NMM was quenched by an internal

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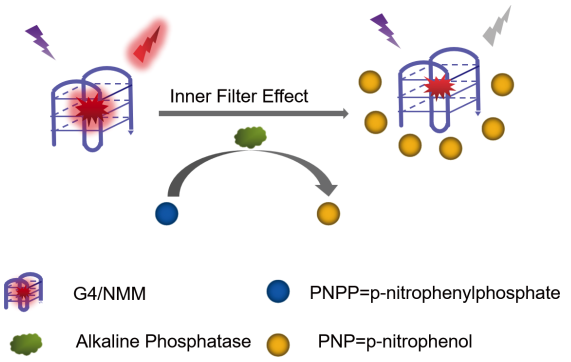
filtration effect. Since ALP was used as a catalyst in this process, an ultra-sensitive biosensor could be built for detecting ALP.

According to the principle, the spectral properties of PNP and G4/NMM were very important. Thus, the UV-vis absorption of PNP and the fluorescence emission spectrum of G4/NMM were analyzed. PNPP, as a control, was also scanned. As previously reported, a new peak appeared at 400 nm when PNPP changed into PNP (Fig. 1A). Coincidentally, the fluorescence spectrum showed the excitation peak of G4/NMM was also 400 nm (Fig. 1B). Thus, in theory, PNP could quench the fluorescence of G4/NMM.



Scheme 1 Schematic diagram of ALP fluorescence biosensor.

For evaluating the feasibility of our design, the fluorescence spectra were measured under different conditions. The G4/NMM complex exhibited strong fluorescence at 610 nm (Black curve in Fig. S1). When PNPP was added, the fluorescence was not affected (Red curve in Fig. S1). However, the fluorescence could be significantly quenched by PNP (Blue curve in Fig. S1). When ALP was added into the mixture of PNPP and G4/NMM, the fluorescence quenching could also be obviously observed due to the hydrolysis of PNPP into PNP (Green curve in Fig. S1). Therefore, these results suggested that the proposed method could be used for detecting of ALP.

The sensitivity of the proposed biosensor was measured by adding different levels of ALP. As shown in Fig. 2A, the fluorescence intensity decreased with the increase of ALP within 0 – 25 U/L. Then, the fluorescence reached a plateau, and the fluorescence change was not significant, even if the ALP concentration continuously increased to 60 U/L. This result suggested that the generated PNP reached saturation. Subsequently, the peak value of the fluorescence was studied for quantitative analysis. As shown in Fig. 2B, the peak intensity showed an excellent linear correlation with ALP in the range from 2.5 to 25 U/L. The calibration equation was $Y = 2028450 - 62375.4834X$ ($R^2 = 0.995$). The detection limit was estimated to be 0.81 U/L based on 3α/slope. Since the normal range of ALP in adult serum was about 40 – 190 U/L,[31] the sensitivity was sufficient to detect ALP in biological samples. In addition,

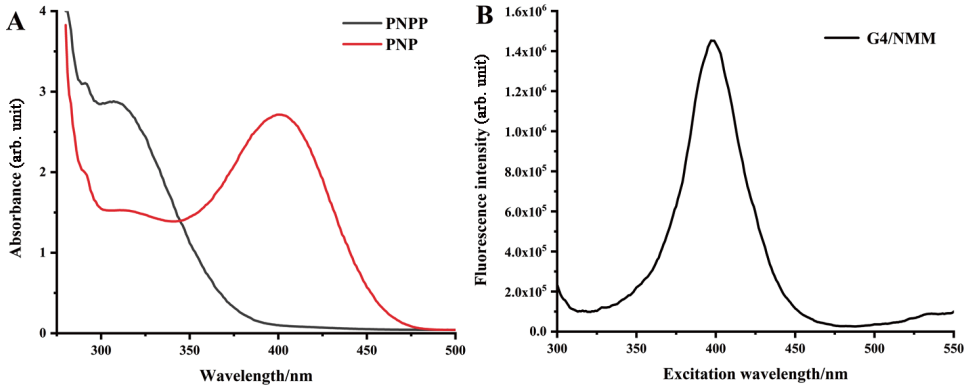


Fig. 1 (A) UV-vis absorption spectra of PNPP and PNP. (B) The fluorescence excitation spectrum of G4/NMM.

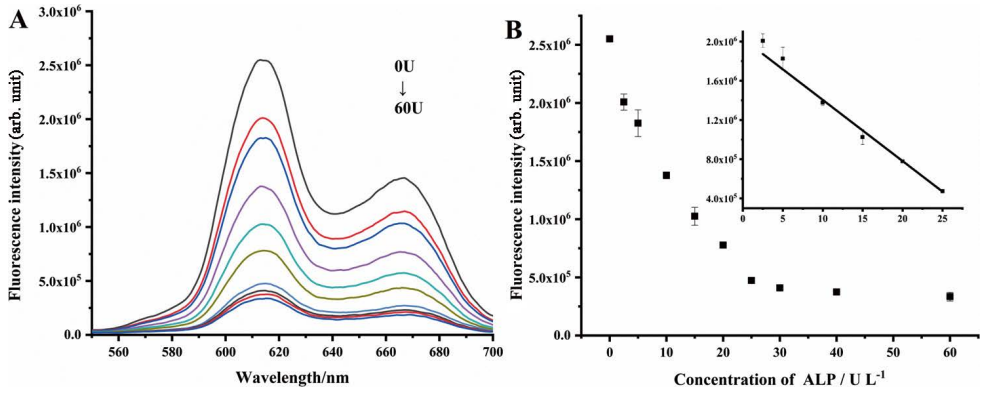


Fig. 2 Sensitivity of the proposed biosensor. (A) Fluorescence spectra of the biosensor response to different concentrations of ALP (0, 2.5, 5, 10, 15, 20, 25, 30, 40 and 60 U/L). (B) The fluorescence signal at 610 nm in different concentrations of ALP. Inset: the fluorescence signal fitted a linear relationship within in the range of 2.5 – 25 U/L. The error bars represent the relative standard deviation in three repetitive measurements.

the analytical performance of the proposed method was compared with that of other reported detection methods (Table S1). The biosensor could be comparable to, or even better than, other methods. These results showed that the proposed biosensor had good potential for ALP detection.

Subsequently, the selectivity of the proposed biosensor was researched by comparing the response of ALP with that of interfering substances (BSA, Glycine, Glucose, Na^+, K^+, Ca^{2+}, Fe^{3+}, Mg^{2+}) which might be present in serum.[33,34] BAS was employed to study the protein interference instead of the human serum protein. The results are showed in Fig. S2. Only ALP could produce a large fluorescence change, while the other interfering substances were all in the background level. These results indicated that the proposed method had high selectivity for the detection of ALP in serum.

In order to estimate the applicability and reliability of the proposed biosensor in real biological samples, the analytical performance of the ALP determination was carried out in bovine serum. Since human serum was difficult to obtain, we used bovine serum to simulate human serum. Before the measurement, the serum samples were treated with a simple 10-fold dilution to reduce interference. Then, different concentrations of ALP were added into the serum samples. Finally, the content of ALP was measured by the proposed biosensor, and the recovery rates were calculated. As shown in Table S2, the recovery rates were between 90.46 and 98.90%, which indicated the reliability of the proposed biosensor to detect ALP in biological samples.

In summary, based on the fact that G4/NMM was a label-free fluorescence reporter with an excellent fluorescence property, and due to the quenching effect of PNP, the fluorescence of G4/NMM dropped dramatically. In this work, using PNPP as a substrate, a novel and highly sensitive fluorescence biosensor for ALP was established based on the internal filtration effect between G4/NMM and PNP. This method was relatively simple, which could be even done in one pot. Meanwhile, it could avoid biological autofluorescence due to the 610 emission wavelength. The titration experiment showed that the linear range of this biosensor was 2.5 – 25 U/L and the detection limit was 0.81 U/L. Compared with other methods for the determination of ALP, this method showed a relatively low sensitivity and avoided the need for complex material synthesis processes. Interference and recovery experiments showed that this biosensor had a great potential in practical diagnostic applications. Besides, the internal filtration effect between G4/NMM and PNP also provided a reference for developing other biosensors.

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Supporting Information

This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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