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A gold nanoparticle-enhanced fluorescence polarization biosensor for amplified detection of T4 polynucleotide kinase activity and inhibition†

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A novel fluorescence polarization (FP) nanosensor based on λ exonuclease cleavage reaction and FP enhancement effect of gold nanoparticles (AuNPs) has been developed for the detection of T4 polynucleotide kinase activity and inhibition. This FP sensor exhibits higher detection sensitivity over traditional fluorescence sensors by two orders of magnitude.

The phosphorylation of the 5′-hydroxyl termini of nucleic acids by polynucleotide kinase (PNK) plays an important role in many important biological processes, such as DNA recombination, replication and repair,[1–3] and was extensively utilized as a powerful tool in bioassays.[4] The development of sensitive and selective techniques capable of screening PNK is of great importance in the field of modern molecular biology research. The typical assay methods for PNK detection were radical isotope ^{32}P-labeling, PAGE and autoradiography.[5–7] However, these methods have the shortcomings of being time-consuming, laborious, not sensitive, or require the radio-labeling of substrates. These limitations are now being addressed by the development of highly sensitive fluorescence assays based on fluorescence quenching or fluorescence resonance energy transfer (FRET).[8–11] For example, a hairpin fluorescence molecular beacon (MB)-based DNA probe combined with the PNK and ligation enzyme-coupled reaction has been developed for real-time investigation of the nucleic acids phosphorylation process.[8] A graphene oxide-based nano-beacon was also used for DNA phosphorylation analysis by Wang and co-workers.[9] Although each method has its advantages, the sensitivities of these methods are still low due to the lack of an amplification mechanism. Thus, the development of more sensitive and convenient methods for PNK assay is still required.

Gold nanoparticles (AuNPs) have attracted significant research interest in bioanalytical applications due to their unique optoelectronic properties and excellent biocompatibility.[12–21] Of particularly interest is the strong enhancement effect of AuNPs, which have been employed to substantially improve the performance and sensitivity of various biosensing systems. Especially, compared with some other particles, such as silicon particles and polymer spheres, AuNPs can easily conjugate with biomolecules *via* self-assembly and retain the biochemical activity of the labeled biomolecules, such as proteins and DNA. Based on the enhanced effect, AuNPs have been used as labels for amplified electrochemical detection,[22,23] quartz crystal microbalance detection,[24–26] chemiluminescence detection[27,28] and resonance Raman scattering-based detection,[29] or as electron relays for the facilitation of interfacial electron transfer on electrodes.[30] Recently, AuNP enhancement functions in network field-effect transistors,[31] in a responsive polymer gel,[32] and in electrical sensors[33] have been reported. More recently, AuNP-enhanced effects in fluorescence polarization (FP) assay of metallic ions,[34,35] endonucleases,[36] and single-nucleotide polymorphism[37] have also been reported. Although many AuNP enhancement-based methods have been reported for sensing of various targets, no such methods are currently available for nucleotide kinase assay.

Herein, we report a novel, highly sensitive and selective FP nanosensor for T4 PNK activity and inhibition by combining λ exonuclease (λ exo) cleavage reaction with the strong FP enhancement effect of AuNPs. By using the AuNP enhancement approach, the detection sensitivity of this method can be significantly improved by two orders of magnitude over traditional assays and FRET methods. The principle of the FP nanosensor is illustrated in Scheme 1. A DNA hairpin probe is modified with AuNPs (44 nm) at the 3′-end, acting as the substrate of T4 PNK. A DNA strand that is partly complementary to the stem of the DNA hairpin is labeled with the fluorescein amidite (FAM) at the 5′-end. λ exo was used for cleavage of the

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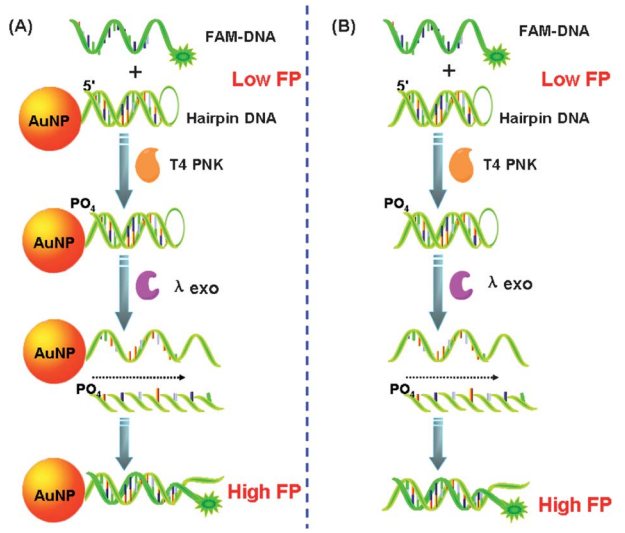

Scheme 1 Schematic illustration of the strategy of T4 PNK activity detection using fluorescence polarization enhancement by gold nanoparticles (A) and without enhancement (B).

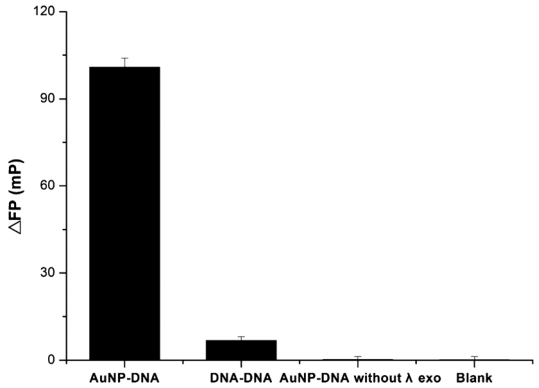

Fig. 1 Fluorescence polarization changes upon addition of T4 PNK at a concentration of 0.1 U mL^{-1}. A blank (FAM-labeled DNA and hairpin DNA without T4 PNK) was used as a control reference.

5′-phosphoryl end product. While the 5′-hydroxyl group of the DNA hairpin associated with AuNPs is phosphorylated by T4 PNK in the presence of adenosine triphosphate (ATP), the resulting 5′-phosphoryl end product was immediately cleaved by λ exo, generating a DNA fragment linked to AuNPs. The FAM-labeled DNA probe is hybridized to the surface of DNA fragment-functionalized AuNPs, forming AuNP-functionalized DNA duplexes. From the Perrin equation,[35] the FP value (*P*) of a fluorophore is proportional to its rotational relaxation time, which depends on its molecular volume. If a fluorescent molecule is free in solution, it rotates at a rate commensurate with its size and the FP value (*P*) is relatively small. However, the fluorescent molecule forms a complex with another substance, its rotational rate decreases and the *P* value increases, and the degree of variation depends on the strength of the binding interaction and the size of the complex.[38,39] In this study, the formation of AuNP-functionalized DNA duplexes results in a substantial increase of the *P* value due to the enlargement of the molecular volume of AuNP-functionalized DNA duplexes. By monitoring the increase in the *P* value, we could detect the T4 PNK activity with very high sensitivity. Of note, the introduction of AuNPs provides significant signal amplification, which improves substantially the performance and sensitivity of the FP sensor for detecting T4 PNK in homogeneous solution.

As a proof-of-principle experiment, a mixture of DNA hairpin-functionalized AuNPs (40 nM DNA) and FAM-labeled DNA probe (40 nM) in Tris–HCl buffer (pH 8.0) containing 10 mM $MgCl_2$ and 12 units of λ exo was placed in two vials with and without T4 PNK. The vials were pre-incubated at 37 °C for 30 min, and the *P* values of the solution were measured. It was found that the *P* value of the solution with T4 PNK was significantly increased compared with that of the solution without T4 PNK (Fig. 1). Control experiments revealed that no changes of *P* values occurred in the absence of λ exo or in the presence of pre-deactivated λ exo. This result indicated that the phosphorylated products were cleaved by λ exo, and then the cleaved products associated with AuNPs formed the duplexes with the FAM-labeled DNA probe. The *P* value of the solution was examined both with and without modification of the DNA hairpin with AuNPs. The *P* value is approximately 15 times higher in the system using the AuNP-functionalized DNA hairpin (AuNP–DNA) compared with the system without AuNP enhancement (DNA–DNA). A substantial increase in the *P* value was attributed to the enlargement of the molecular volume of the AuNP-functionalized DNA duplexes with slow rotation. These facts represent a remarkable AuNP enhancement function for FP assay of T4 PNK activity.

The binding of the FAM-labeled DNA probe to the surface of functionalized AuNPs after the T4 PNK and λ exo-coupled reaction was verified by fluorescence measurements. The AuNP-functionalized DNA hairpin complexed with T4 PNK and λ exo was incubated with the FAM-labeled DNA probe for various elongation times. Afterward, the DNA–AuNP conjugates were centrifuged and subsequently fluorescence was measured. The fluorescence spectra of the resulting conjugates showed a fluorescence band centered at 520 nm. As the reaction time was prolonged, the intensity of this band increased (Fig. S1†). In contrast, the control experiment measured under otherwise identical conditions but in the absence of T4 PNK did not show any observable fluorescence at 520 nm. The result demonstrated that the FAM–DNA probe could be hybridized to the surface of functionalized AuNPs after the T4 PNK and λ exo-coupled reaction. This fact provides a solid foundation for the present method.

In order to improve the sensitivity of T4 PNK detection, the relevant experimental parameters affecting FP assay for T4 PNK were assessed and optimized. The reaction time is a crucial parameter for the T4 PNK-catalyzed phosphorylation and the coupled λ exo cleavage process. As shown in Fig. S2,† the *P* value increased gradually with the increase of the reaction time, and then reached equilibrium after 30 min, suggesting the complete phosphorylation and cleavage process. Thus, 30 min was selected as the optimal time for the reactions. The amount of λ exo used for the cleavage process was also investigated. The *P* value increased dramatically with the amount of λ exo up to

12 units and then reached equilibration over 12 units (Fig. S3†). Hence, 12 units of λ exo were selected for the subsequent assay. Additionally, the T4 PNK and λ exo coupled reaction is also sensitive to pH and the concentration of Mg^{2+}. The experimental results showed that the optimal pH value and the concentration of Mg^{2+} for the coupled enzyme catalyzed procedure were 8.0, and 10 mM, respectively (Fig. S4†). Moreover, the size of the AuNPs modified to the DNA significantly affected the *P* values. Increasing the diameter of the AuNPs (13, 25, 30, 38, and 44 nm) changed the *P* values more obviously when the same concentration of T4 PNK was used (Fig. S5†). Therefore, 44 nm AuNPs were used in this study.

To evaluate the sensitivity of the AuNP-enhanced FP sensor, we tested the response of the assay on different concentrations of T4 PNK from the same stock solution. As shown in Fig. 2A, the *P* value increased as the concentration of T4 PNK increased. The present limit of detection for this method is 1.0×10^{-5} U mL^{-1}, which is about four orders of magnitude lower than that of the traditional radical isotope ^{32}P-labeling methods.[40] Also, the sensitivity of this method is two orders of magnitude higher than general fluorescence methods.[8–11] The results also show a wide detection range for T4 PNK from 1.0×10^{-5} to 50 U mL^{-1}. In contrast, when the FP sensor without AuNP enhancement was applied to detect T4 PNK, a sensitivity that corresponds to a T4 PNK concentration of 0.1 U mL^{-1} was observed (Fig. 2B). The fluorescence lifetime (τ) of FAM-labeled DNA before and after binding with functionalized AuNPs (that is, before and after enzymatic reaction) was measured (Fig. S6†). It was found that $\tau = 3.59$ ns for FAM before FAM-labeled DNA binding with functionalized AuNPs, and $\tau = 3.57$ ns for FAM after FAM-labeled DNA binding with functionalized AuNPs. This result indicated that the binding of FAM-labeled DNA on the AuNP surface did not obviously influence the lifetime of FAM. Thus, the lifetime of FAM in our system influenced the FP slightly. From the Perrin equation,[35] we could conclude that the substantial sensitivity improvement of the AuNP-enhanced sensor was mainly attributed to the enhancement of the probe molecular volume.

The AuNP-enhanced FP biosensor developed here could also be used for screening the inhibitors of T4 PNK. To evaluate this property, we investigated the inhibition effects of adenosine diphosphate (ADP), ammonium sulfate ($(NH_4)_2SO_4$), and sodium hydrogen phosphate (Na_2HPO_4) on the activity of T4 PNK. Because there were two enzymes involved in this reaction system, it was necessary to eliminate the effect of inhibitors on λ exo before studying the effect of inhibitors on the activity of T4 PNK. The control experiments were performed by the phosphorylation of the AuNP-functionalized DNA hairpin probes at first, followed by addition of λ exo and inhibitors. It was found that all inhibitors tested had no influence on the activity of λ exo for the concentrations of the inhibitors up to 80 mM (Fig. S7†). For comparison of the inhibition abilities of the inhibitors, the influence of these inhibitors at 2 mM each on the activity of T4 PNK was directly measured. The inhibitors affected T4 PNK differently. As shown in Fig. 3A, the inhibition of ADP was the most effective, reaching an inhibition ratio of 62%. Ammonium sulfate was the second effective, much more so than sodium hydrogen phosphate. We also investigated how the concentration of ADP influenced the activity of T4 PNK. The results obtained are shown in Fig. 3B. It was very clear that the inhibition was enhanced with the increase of the ADP concentration. These results suggest the potential use of the AuNP-enhanced FP biosensor for studies of PNK inhibition.

In summary, we have developed a novel AuNP-enhanced PF nanosensor for rapid and sensitive detection of the activity and

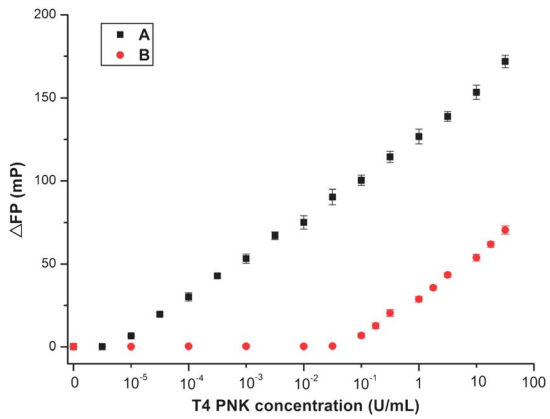

Fig. 2 Plots of fluorescence polarization changes as a function of T4 PNK concentrations for the AuNP–DNA (A) and DNA–DNA system (B).

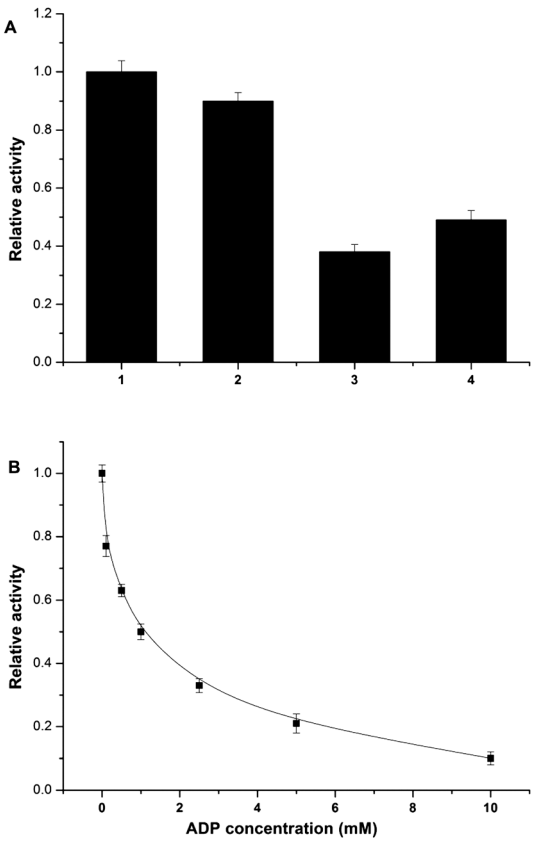

Fig. 3 (A) Influence of different compounds on the activity of T4 PNK. (1) No inhibitors, (2) Na_2HPO_4, (3) ADP, and (4) $(NH_4)_2SO_4$. (B) The inhibition of different concentrations of ADP on the activity of T4 PNK.

inhibition of PNK. The method takes advantages of λ exo cleavage reaction and the strong enhancement function of AuNPs. In contrast to traditional methods, the present method uses non-isotopic substrates and a simpler procedure in which the enzyme assay is conducted in homogeneous solution, without separation and other troublesome procedures. Additionally, this assay has very high sensitivity, which is more than two orders of magnitude higher than that of traditional methods and general fluorescence methods. Moreover, this method is based on the FP enhancement, therefore, it is amenable to be carried out in 96- or 384-well plates, rendering it suitable for routine high-throughput applications. Given the crucial roles of kinases in some biological processes, this AuNP-based FP sensing platform is promising in developing high-throughput assays for drug discovery and clinical diagnostics.

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