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Phosphorylation triggered poly-nanoparticle assembly for naked-eye distinguishable T4 polynucleotide kinase detection†

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A novel naked-eye distinguishable nanosensor based on phosphorylation triggered poly-nanoparticle assembly strategy has been designed for detecting T4 PNK activity. This colorimetric sensor exhibits conveniently homogeneous operation with simple, sensitive and easily scalable properties.

It is well known that various exogenous and endogenous agents including chemical substances,[1] ionizing radiation,[2] as well as nucleases[3] may lead to DNA damage. DNA strand break, as a typical DNA damage example, often results in DNA strands with 3′-phosphate and 5′-hydroxyl termini.[4] However, almost all of DNA polymerases and ligases characterized are highly selective for the type of DNA ends, and require 5′-phosphate and 3′-hydroxyl DNA temini.[5–7] T4 polynucleotide kinase (T4 PNK), which was first discovered by Richardson in 1965, just has the ability to catalyze the transfer of the γ-phosphate group from adenosine triphosphate (ATP) to the 5′-hydroxyl end of DNA or RNA as well as the dephosphorylation of the 3′-phosphate group of polynucleotide, that are then suitable for sealing by DNA ligase.[8–10] Accordingly, T4 PNK is thought to play a key role in the modification of damage-induced DNA termini in human cells,[11] and sensing PNK activity is therefore of great importance.[12] Traditional methods like radical isotope ^{32}P-labeling, PAGE and autoradiography were reported for PNK analysis.[5,8,11] Unfortunately, these approaches are discontinuous, laborious, costly and might be harmful to human health.[13,14] In recent years, to avoid those problems, fluorescent,[13,14] electrochemical,[15,16] colorimetric,[9] DNAzyme-based[17] and nanochannel[18] biosensors have been developed in this area. Such assays were effective, but technically demanding, quite expensive and sometimes largely influenced by many environmental factors.[12,18,19] Therefore, more avenues still need to be explored to have a convenient, rapid, sensitive and accurate method to measure the activity of PNK.[18] Notable examples include work reported by He and co-workers, who have utilised transition metal complexes-based luminescence to develop label-free assays for PNK activity.[20] By comparison, the method here was based on a simply colorimetric way.

Gold is the first metal to be transformed into a colloidal state. Its simple synthetic procedure, high extinction coefficients and large surface area make it highly conspicuous in the area of DNA sensing recently.[21] Inspired by these advantages, different methods of PNK detection by using gold nanoparticles also have been proposed in recent years. However, the nanoparticles were usually used as signal enhancers but detectors in those methods. For examples, Wang utilized the 5′-phosphate DNA modified gold nanoparticles and the TiO_2 nanotubes in order to generate a novel electrochemical sensor for the detection of T4 PNK with Au nanoparticles amplification.[15] Huang presented a fluorescence polarization nanosensor based on λ exonuclease cleavage reaction and fluorescence polarization enhancement effect of gold nanoparticles.[22] Although these methods were quite effective, they still depended on professional signal detection equipments. After Mirkin and co-workers reported the breakthrough method for detecting polynucleotides which utilizes the distance-dependent optical properties of aggregated gold nanoparticles functionalized with oligonucleotides,[23,24] the development of DNA-functionalized gold nanoparticles has been advanced by leaps and bounds. Previous work has demonstrated that large nanoparticles network formed through DNA strands hybridization or ligation can be distinguished by the naked eye.[23] In addition, due to the obvious advantages such as larger touch surface, more even reaction and requiring no complex rinse and separation, it seems that homogeneous reaction has an advantage over solid phase reaction. Therefore, it has a wide foreground to introduce

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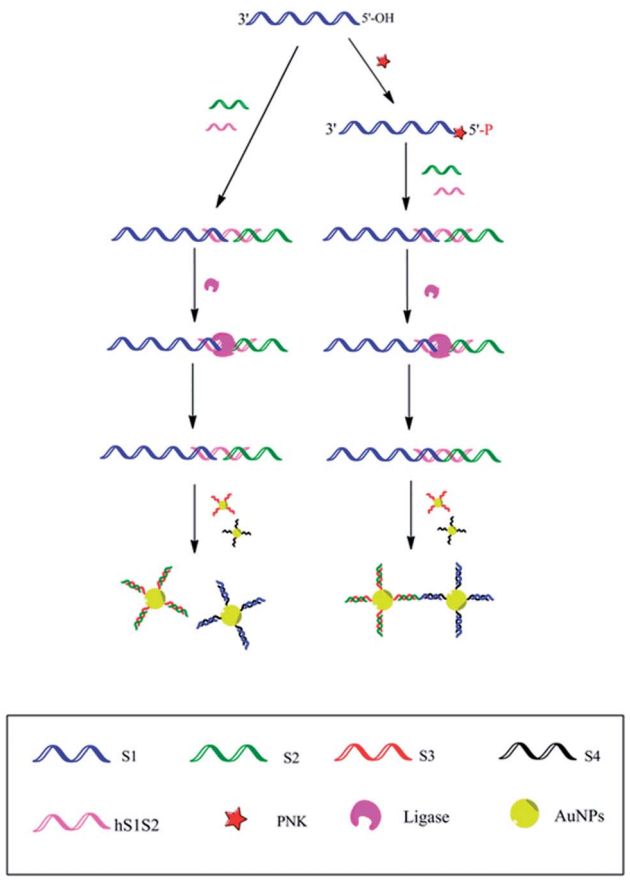
the homogeneous reaction in PNK detection system. Here, we have utilized the property of DNA–gold nanoparticles self-assembly to create a simple building block and exploited a homogeneous, highly specific, sensitive, convenient and naked-eye directly identified T4 PNK detection system which has not been reported thus far.

The detection method is illustrated in Scheme 1. The phosphorylation triggered multi-nanoparticle assembly strategy has been designed for detecting T4 PNK activity. There are five sequences (Table 1), two of them (S3 and S4) were immobilized on the surface of gold nanoparticles by strong sulfur–gold adsorption. S1 acting as a target recognition probe and S2 as a common probe, are both designed to be perfectly complementary to S4 and S3 respectively. In the present of T4 PNK, the 5′-hydroxyl termini of S1 can be phosphorylated, and the subsequent ligation process mediated by T4 DNA ligase will process and produce a complete long strand S12. Hybridization of the newly produced oligonucleotide S12 with the two probes on gold nanoparticles results in a gradual color fade and a precipitation reaction ensues within 30 minutes. In order to improve the efficiency and specificity of ligation, hS1S2 is introduced as a helper strand which contains a 5-base-pair overlap sequence with S1 and another 5-base-pair overlap with S2. The 10-base oligonucleotide closes S1 and S2 through hybridization and causes the relatively high efficiency of the two adjacent probes covalently joining by T4 DNA ligase. Since the probe S2 also carries the 5′-hydroxyl termini, it will be added into the reaction solution after S1 was phosphorylated by T4 PNK to avoid the undesired target-catalyzed phosphorylation. In the assembly step, S3 and S4 have much more complementary bases with S12 than hS1S2 so that ssDNA S12 prefers to hybridize with the probes on the nanoparticles and forms a more stable double strand, rather than with the helper hS1S2.[25] Nevertheless, in the absence of T4 PNK, S1 keeps its 5′-hydroxyl termini so that T4 DNA ligase cannot perform its function. Although S1 and S2 can still hybridize with S3 and S4, but never link together. Accordingly, it can hardly form the poly-DNA–gold nanoparticle assembly.



Scheme 1 Sketch to the concept and the experimental principle of PNK detection using gold nanoparticles.

We first validated the feasibility and effectiveness of the T4 PNK detection system by directly colorimetric response in Fig. 1A. The left sample of the photograph shows distinctly polymeric gold nanoparticle–polynucleotide aggregate and highly transparent, colorless supernatant in standardized detection system. It indicates the successful phosphorylation of the target recognition probe by T4 PNK. In contrast, no apparent precipitation is observed in the middle and right ones even after prolonged storage, which represent adding inactive PNK and without PNK respectively. It has proved that this analytical assay is as simple and effective as we initially predicted. Furthermore, it is worthwhile to note that most of the previous works showed a red to blue or gray color change in their experimental solutions during detection process, which are different from our purplish red aggregates.[24,26–31] Since nanoparticle aggregates with interparticle distances substantially greater than the average particle diameter appear red, but as the interparticle distances in these aggregates decrease to less than approximately the average particle diameter, the color becomes blue.[24,32,33] We can imply that the interparticle distance is still slightly greater than or close to the average particle diameter here.

As is well known, in most systems, the gold nanoparticles in solution were stabilized by the adsorbed negative ions (citrate). Their repulsion prevented the strong van der Waals attraction between gold nanoparticles from causing them to aggregate. However, high concentration of salts will screen the charge on the surface of gold nanoparticles, resulting in the gold surface plasmon state change, and color becomes blue and nanoparticles aggregate.[34,35] This would be difficult to distinguish whether the color change is caused by the target or high ionic

Table 1 The DNA sequences used in this study and their abbreviations

DNA code	DNA sequence
S1	5′-GCA GGA CCA TTC TTT GAT ACA GA-3′
S2	5′-ACT AGC TAC GAT-3′
CK	5′-ACT AGC TAC GAT GCA GGA CCA TTC TTT GAT ACA GA-3′
S3	5′-C_6S-S-TCT GTA TCA AAG AAT GGT CCT GC-3′
S4	5′-ATC GTA GCT AGT-C_3S-S-3′
hS1S2	5′-CCT GCA TCG T-3′

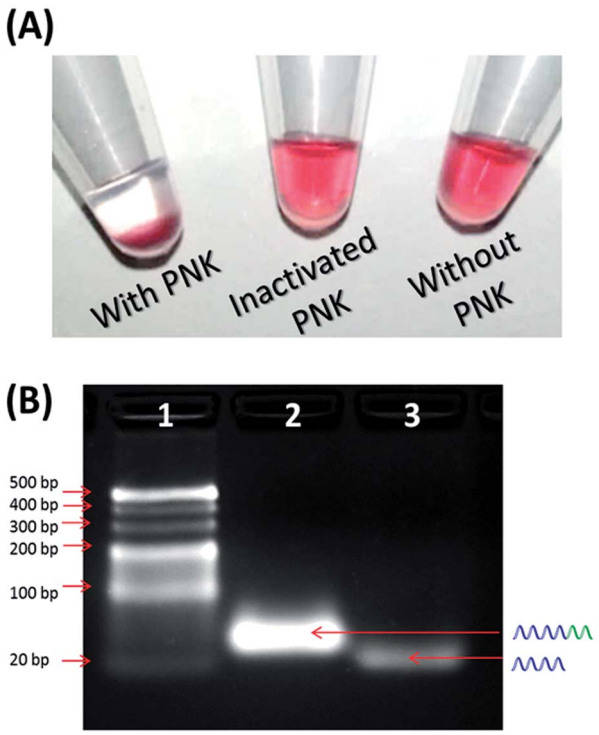


Fig. 1 (A) Photograph showing colorimetric response of a PNK detection system. Distinctly aggregate and transparent supernatant can be observed in the presence of the PNK. While, the inactive PNK and without PNK samples keep the red uniform solution. (B) Agarose gel separated products obtained from a phosphorylation and ligation experiment. Lane 2: in the presence of T4 PNK. Lane 3: in the absence of T4 PNK.

strength. Fortunately, the result of our detection system is credible, regardless of considering the problems above, because the DNA hybridization just leads to the precipitate but color change.

Moreover, the phosphorylation process is further confirmed by analyzing the reaction products in an agarose gel electrophoresis (AGE), Fig. 1B. The slowly and rapidly migrating nucleotide fragments have appeared in lane 2 and lane 3, which displayed the ligation results of weather phosphorylated or not respectively. The results clearly indicate that T4 PNK acts as a necessary prerequisite for ligation during repair, inducing T4 DNA ligase repairing DNA nicks and forming a complete longer probe. The insensitivity of AGE allows the palpable observation of DNA bands that are longer than about 20 bases. There is a clear single band of the ligated S12 with helper hS1S2 in lane 2. Consequently, for the failure ligation sample, nothing but the band of S1 (23 bases) will be seen in lane 3 (S2 only has 12 bases). In addition, the band in lane 2 is much brighter than the one in lane 3. We suspected the explication that SYBR Green I presents much higher affinity to double strands than single strand DNA. As joining of nicks by DNA ligase, the hybridization of helper sequence hS1S2 with S12 is more stable and the duplex contains 10 complementary base pairs. Otherwise hS1S2 may be released from the two shorter strands (S1 and S2) during the electrophoresis process, and only the single strand S1 can be seen in gel.

To verify the accurate wavelength for UV-vis measurement as well as the positive control purpose, a single strand DNA (CK), whose sequence is same with the ligation product S12 was designed. As shown in Fig. 2A, the absorbance at 524 nm dramatically decreased with about 20 nm red shift after the addition of CK to the gold nanoparticles collosol directly, which implys the successful hybridization between CK and S3, S4, creating poly-gold nanoparticle assembly.

After having determined the measuring wavelength (524 nm), the kinetics of this assay was followingly explored. The absorbance of each sample was monitored and the time courses were plotted in Fig. 2B. Expect for time gradients, further control experiments were also conducted. The positive control was investigated with CK instead of the ligated S12. Pure water or an inactivated T4 PNK (heated in 75 °C for 10 min) replaced the standardized T4 PNK as two negative controls. As illustrated above, different phosphorylation times showed different kinetic behavior to gold nanoparticle assembly. The absorbance of two negative controls remained approximately constant during whole detection period. Interesting, fairly fast absorbance decreases were revealed for the positive control and most of the phosphorylated products expect for the half hour one, which implys that the successful "phosphorylation and ligation" process of S1 and S2 in the presence of T4 PNK is performed. However, half hour reaction time may be too hasty to

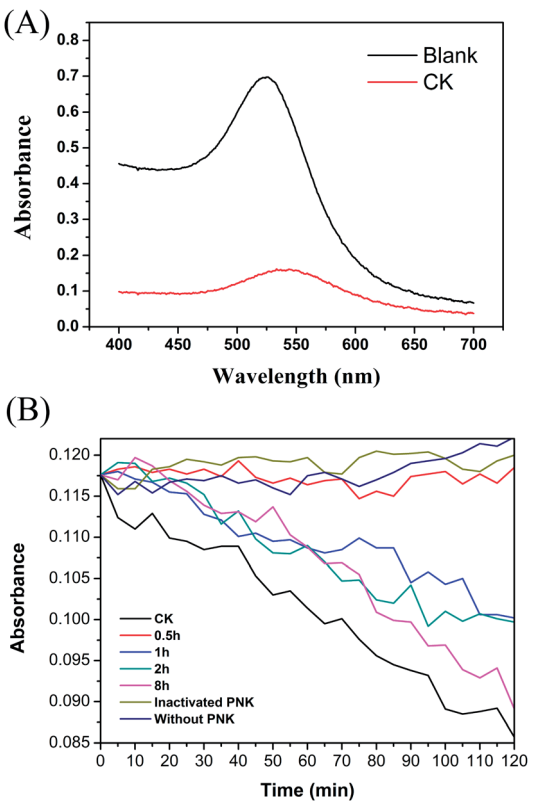


Fig. 2 (A) UV-vis spectra for gold nanoparticles in the presence of CK or not. (B) Monitoring of the phosphorylation reactions in real-time: 0.5 h, 1 h, 2 h, 8 h, positive control (CK), negative controls (inactive PNK and without PNK).

phosphorylate enough substrates for linking the gold nanoparticles and 8 hours one seems too long in this system, yet one to two hours are sufficient.

Fig. 3A displayed the UV-vis spectra of the sensing system following the successive addition of T4 PNK. As expected, a gradual decrease in absorbance signal was clearly observed along with the increase of T4 PNK concentration ranging from 0.0385 to 1.3533 U mL^{-1}. It produced a good calibration curve relationship and, more important, the novel method was conducted in PCR tubes which facilitated the operation and was easily available.

It is generally known that a number of factors are likely to influence the activity of the T4 PNK reaction.[15,36] Since T4 PNK catalyzes the transfer of the γ-phosphate from ATP to the 5′-OH group of DNA, it is necessary to investigate the influence of ATP concentration on the rate of reaction here. As shown in Fig. 3B, the increase of ATP concentration resulted in the reducing of absorbance responses in low concentration. While a gradually rise was observed when the concentration of ATP continued to increase. This slight inhibition effect is probably due to the competitive binding to T4 PNK between DNA and ATP. When the concentration of ATP is relatively high, the binding sites of T4 PNK for 5′-hydroxyl DNA are partially blocked. Kinetic analysis favors a sequential mechanism in which both ATP and the 5′-OH polynucleotide bind the enzyme prior to dissociation of either product.[15,37] Consequently, 0.69 mM was considered as the optimal ATP concentration to obtain a high sensitivity in this detection.

The hS1S2 serves as a helper strand which closes S1 and S2 through hybridization to facilitate the junction of nicks by T4 DNA ligase. In order to study the influence between hybridization effect and ligase efficiency, AGE was run to explore the optimized hybridization and ligation condition (Fig. 3C). The working temperatures of hybridization and ligation for the samples from lane 2 to 7 are 4 °C and 4 °C, 4 °C and 22 °C, 4 °C and room temperature, room temperature and 4 °C, room temperature and 22 °C, room temperature and room temperature, respectively. Since ligation produces a significant shift in DNA electrophoretic mobility, the bands with lower mobility appear in the lane 2, 3, 5 and 6 (the bands of lane 4 and 7 are failed for ligation). A relatively brighter band was observed in lane 5, reflecting the relatively better condition for ligation, that is, hybridization in room temperature and ligation in 4 °C overnight. Besides, hybridization in room temperature and ligation in 22 °C for 1 h in lane 6 also played a good effect next to lane 5. At the same time, the effect of T4 DNA ligase was also investigated by AGE. As shown in Fig. 3D, increasing concentrations of T4 DNA ligase greatly promoted the yield of this

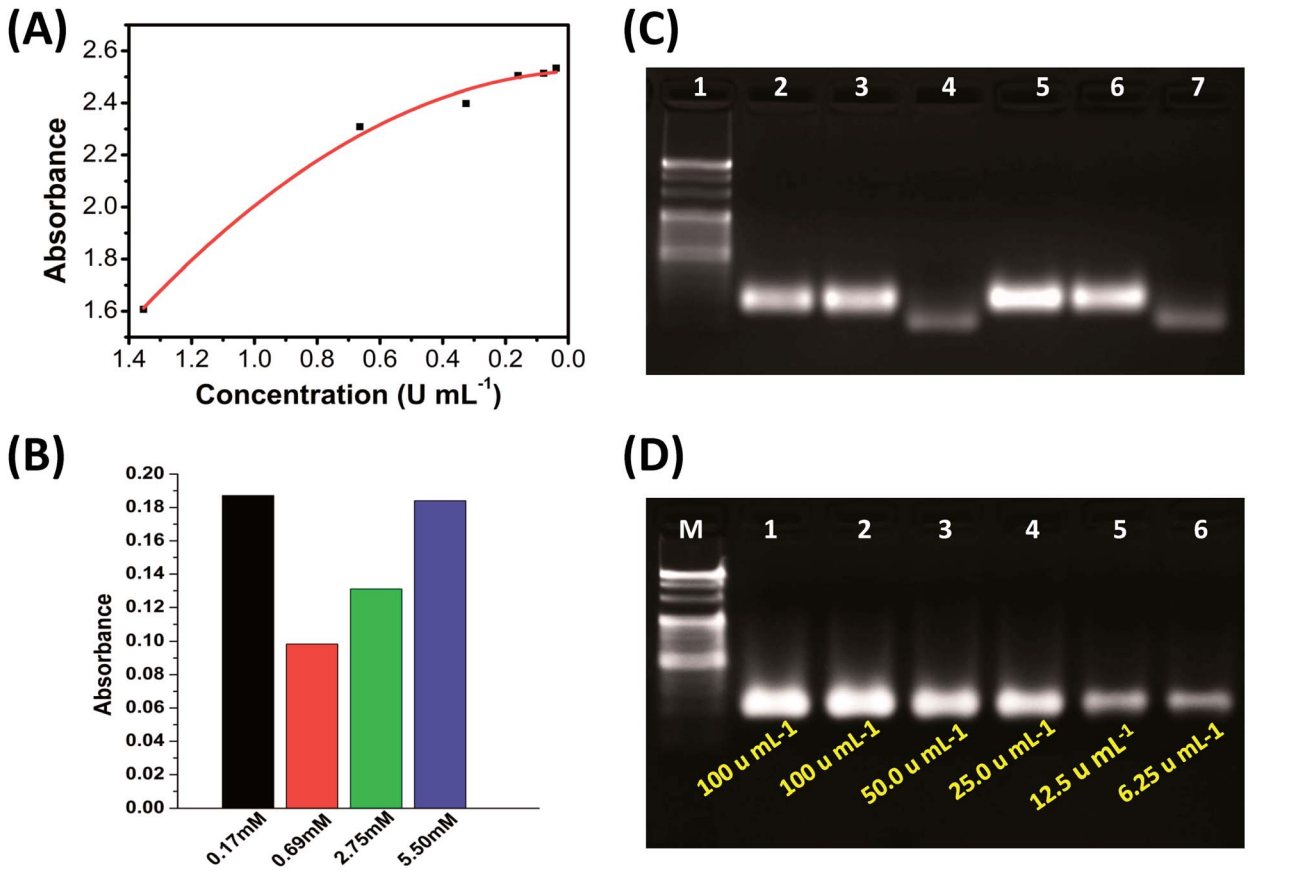


Fig. 3 (A) Calibration curve for the absorbance *vs.* concentration of T4 PNK. (B) Effect of ATP concentration on T4 PNK activity. (C) Effect of helper hybridization and ligation on detective system. (D) Effect of T4 DNA ligase concentration on detective system.

reaction. It can be seen that the higher the concentration of ligase, the brighter the band. It is probable that addition of enough ligase may guarantee the ligase could efficiently repair DNA nicks.

Conclusions

In summary, a handy and visible nanoparticle-sensor is demonstrated to monitor the activity of T4 PNK in PCR tubes. This sensing strategy relies on the colorimetric fading of gold collosol by T4 PNK triggered ligation and hybridization-created assembly of gold nanoparticle building block. Compared with the traditional methods, no lock operations are needed in this system and no instrument is needed, if we don't want an accurate quantity, that is, it can be detected by naked-eye directly. And the method is based on the colorimetric change, therefore, it is amenable to be carried out in 96- or 384-well plates, rendering it suitable for routine high-throughput applications. This makes the assays more flexible and easily scalable, which is promising in developing high throughput assays for medical diagnosis and drug discovery even in future daily life. Moreover, it is conceivable that this method may be also used for parallel assays of hundreds of samples, such as other enzymes and some biological small molecules (ATP *etc.*), as desired in clinical applications.

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