Supplementary Material (ESI) for Chemical Communications


# Enhanced chemiluminescence detection of thrombin based on cerium oxide nanoparticles

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## Experimental Section

**Chemicals:** All oligonucletides designed according to the literature in the present study were purchased from SBS Genetech Co., Ltd. (Beijing, China), and their sequences were as follows. Thrombin aptamer ($S_1$): 5'-$NH_2$-$(CH_2)_6$-TTT TTT TTT GGT TGG TGT GGT TGG-3', reporter probe ($S_2$): 5'-SH-$(CH_2)_6$-TTT TTT ACA CCA ACC-3', signal probe ($S_3$): 5'-SH-$(CH_2)_6$-TTT TTT TT-$NH_2$-3'. $Ce(NO_3)_3 \cdot 6H_2O$ and polyacrylic acid (PAA) were from Kemiou Research and Development Centre of Chemical Regent (Tianjin, China).

Thrombin, human serum albumin (HSA), immunoglobulin G (IgG), transferrin, lysozyme, fibrinogen, antithrombin III, prothrombin, and lysozyme were from Sigma (St. Louis, MO), and used without further purification. Other chemicals employed were all of analytical grade and triple distilled water was used throughout. The human plasma samples were provided by the Medical School Hospital of Qingdao University (Qingdao, China). A luminol (standard powder, Sigma-Aldrich) stock solution ($5.0\times10^{-2}$ M) was prepared by dissolution in 0.5 M NaOH and further stored in dark. The stock solution was consecutively diluted with 0.1 M NaOH/$NaHCO_3$ to the proper solution used for CL determination. MBs modified with carboxyl groups (0.5-1.0 μm, 10 mg $mL^{-1}$) and magnetic rack were obtained from BaseLine Chrom Tech Research Centre (Tianjin, China). All the reagents used were analytical grade and double-distilled water was used throughout.

**Instrumentation:** The CL measurements were performed with a BPCL ultraweak luminescence analyzer (Institute of Biophysics Academic Sinica, Beijing, China). Transmission electron microscopy (TEM) was taken with JEM-2000EX/ASID2 instrument (HITACHI, Japan). UV/Vis spectra were carried out on a Cary 50 UV/Vis/NIR spectrophotometer (Varian). The CL

spectra were measured on a model FL 4500 spectrofluorometer (HITACHI) with the excitation light source being turned off.

**Preparaton of AuNPs:** Gold nanoparticles were prepared by citrate reduction of $HAuCl_4$ according to the reference.[1] Briefly, 10 mL of 38.8 mM sodium citrate was immediately added to 100 mL of 1.0 mM $HAuCl_4$ refluxing solution under stirring, and the mixture was kept boiling for 15 min. When the solution turned to a wine red, it was stirred for another 10 min. Then it was cooled to room temperature with continuous stirring. The sizes of the AuNPs were verified by TEM, and UV/Vis spectrum was also recorded (See Figs. S8 and S9).

**Preparation of PNCs:** PNCs were prepared according to the reference.[2] 5.0 mL solution of 1.0 M $Ce(NO_3)_3$ was mixed with 1.5 mL PAA. The mixture was added to 36.0 mL ammonium hydroxide (30%) solution. Vast white precipitate was appeared, shaking the breaker to make it dispersed. The mixture was then stirred continuously for 24 h. The solution color turned from yellow to deep brown, indicting the formation of PAA coated $CeO_2$ nanoparticles. The solution was centrifuged at 4000 rpm for 30 min, the precipitate was washed, recentrifuged, and dispersed in 1 mL of double distilled water (DDW) stored at 4 ºC.

The preparation of cerium oxide nanoparticles without PAA modification was the same except that PAA was not used.

**Preparation of AuNPs Labeled with PNCs and signal and reporter DNA probes:** Preparation of bio-bar-coded AuNPs containing PNCs was performed according to our previous report.[3] Briefly, the mixture of $6.0\times10^{-11}$ mol of $S_2$ and $1.4\times10^{-10}$ mol of $S_3$ was activated with acetate buffer (pH 5.2) and 5 μL of 10 mM TCEP for 1 h, and then added to 1 mL of freshly prepared AuNPs and shaken gently overnight. After reaction of 16 h, the DNA-AuNP conjugates

were aged in salts (0.01 M NaCl, 5.0 mM tris-acetate buffer) for another 40 h. Excess reagents were removed by centrifuging at 15,000 rpm for 30 min. The red precipitate was washed, recentrifuged, and dispersed in 1 mL of buffer containing 300 mM NaCl, 25 mM tris(hydroxymethyl)aminomethane (Tris) acetate, pH 8.2.

A 200 μL of 0.1 M imidazole solution (pH 6.8) was added to 1.0 mL of the prepared oligonucleotide-functionlized AuNP solution. After 30 min, 100 μL of 0.1 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide solution (EDC) solution and 0.56 mL of PNCs were added to the mixture and incubated at room temperature for 12 h with gentle shaking. Excess reagents were removed by centrifugation at 10,000 rpm for 30 min. The precipitate was washed and then dispersed into water. The solution of AuNPs bioconjugates was stored at 4 °C for the hybridizations.

**Preparation of thrombin aptamer-immobilized MBs:** A suspension of carboxyl MBs (50 μL) was washed three times with 0.01 M imidazol-HCl buffer (pH 6.0, 3×100 μL). A 0.1 M EDC solution (100 mL) was added to the MB solution and the mixture was incubated at room temperature for 20 min to activate the carboxylate groups on the MBs. Then, $1.8\times10^{-10}$ mol $S_1$ was added and the resulting mixture was stirred for 1 h. After magnetic separation, MBs were washed three times with buffer (7 mM Tris-HCl, pH 8.0, 0.5 M NaCl). The washed MB/$S_1$ was resuspended in 200 μL PBS solution and incubated with AuNPs labeled with PNCs and probes at 37 °C for 30 min. After magnetic separation to remove excess AuNP bioconjugates, the modified MBs were resuspended in 20 μL of the above buffer before use.

**Fabrication of Sensing Assay of Thrombin:** 50 μL of modified MBs and 75 μL of different concentrations of thrombin were mixed, and the final volume was adjusted to 2.0 mL with 0.1 M

PBS buffer containing 1.0 mM $Mg^{2+}$ (pH 7.3). After incubated for 30 min at 37 °C, the supernatant was for the following CL measurements. A 400 mLof the solution was introduced into a quartz cuvette containing 500 μL of 3.0 μM $H_2O_2$, and was diluted to 2 mL with BR buffer. A 200 μL of the resulting soluiton was then introduced into a quartz cuvetteand and the CL signal was measured in the luminescence analyzer with injecting of 200 μL of $5.0\times10^{-4}$ M luminol in 0.1 M NaOH/$NaHCO_3$. Seven other proteins, such as HAS, IgG, transferrin, lysozyme, fibrinogen, antithrombin III, and prothrombin, were analyzed separately to test the specificity of the assay, using the same procedures as those used for human α-thrombin.

**Plasma Samples Treatment:** lasma samples were pretreated as the same as in the previous reports.[3] Salt solution (2 M ammonium sulfate and 0.1 M NaCl) was added to the plasma samples, and the mixture was then immediately centrifuged for 5 min at 10000 rpm at 4 ºC. The plasma was removed and frozen at -70 ºC until used. Different concentrations of standard solutions of thrombin were spiked into the pretreated plasma. Thrombin was measured after appropriate dilution and was calculated from the measured concentrations after correction for dilution.

## Results and Discussion

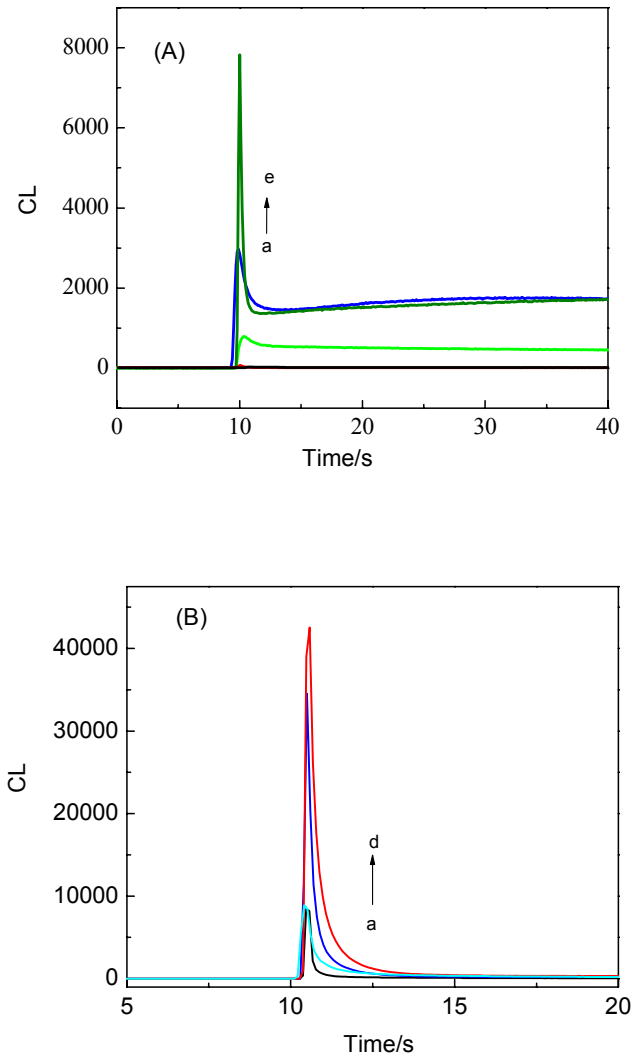



**Fig. S1** (A) Kinetic curves of luminol CL reactions of (a) luminol-$Ce(NO_3)_3$, (b) luminol-PNCs, (c) luminol-$H_2O_2$, (d) luminol-$H_2O_2$-$Ce(NO_3)_3$, and (e) luminol-$H_2O_2$-PNCs. (B) CL spectra for luminol–$H_2O_2$ system with (a) cerium nanoparticles without PAA, (b) PNCs(b), (c) $Co^{2+}$, (d) $Co^{2+}$ and PNCs. The conditions: $5.0\times10^{-4}$ M luminol in 0.1 M NaOH–$NaHCO_3$ buffer solution, 3.0 μM $H_2O_2$, 1.0 μM PNCs in 20 mM BR buffer (pH 4.2), the concentrations of $Ce(NO_3)_3$ and $Co^{2+}$ were of 1.0 μM and 60 μM, respectively.

In order to verify whether the PNCs absorb the energy of luminol derivative, a series of experimentals have been performed as shown in the Fig. S1B. The CL of luminol-$H_2O_2$ system was tested with PNCs (curve a) and cerium oxide nanopartcles without PAA modification (curve b), respectively. The two CL intensities were comparable with each other, indicating that the PAA had no effect on luminol derivative. $Co^{2+}$ was used as control to test whether the PNCs have luminol absorbance. When the luminol-$H_2O_2$ system was catalysed with $Co^{2+}$, the CL intensity was arround 25000. When the luminol-$H_2O_2$ system was catalysed simultaneously with $Co^{2+}$ and PNCs, the CL intensity increased, indicating that PNC has no luminol absorbance. From the comparison of the CL catalyzed with $Co^{2+}$, $Co^{2+}$+PNCs, cerium oxide nanopartcles without PAA and PNCs, one can see that neither the cerium nanoparticles nor the polyacrylic acid are acting as a sink to absorb some of the energy.

**Optimization of the CL reaction conditions.**

Effects of concentrations of luminol, $H_2O_2$, PNCs were investigated. The results in Fig. S2 showed that the CL intensity increased with increasing luminol concentration from $3.0\times10^{-4}$ M to $7.0\times10^{-4}$ M, and reached the highest at $5.0\times10^{-4}$ M (Fig. S2a). In the range of $3.0\times10^{-7}$ M to $7.0\times10^{-6}$ M for $H_2O_2$ (Fig. S2b), the peak was good. CL signals were also increased with $H_2O_2$ concentration increasing, and decreases were obtained when the $H_2O_2$ concentration was larger than $3.0\times10^{-6}$ M. Therefore, the optimized concentrations for luminol and $H_2O_2$ were $5.0\times10^{-4}$ M and $3.0\times10^{-6}$ M.

As a critical factor for the CL system, the effect of pH values of luminol and PNC solutions were also studied. For luminol solution, the pH value for luminol in the range of 9.0 to 13.0 were

tested, and the results demonstrated that using luminol prepared by 0.1 M NaOH-$NaHCO_3$ buffer with pH 11.0 was the optimized value (Fig. S2c). Since PNCs have strong catalysis activity at acidic pH values, the CL intensity was studied with pH 3.0-5.1. From Fig. S2d, one can see that when the pH of PNC solutionS was lowere than pH 4.2, the CL intensity increased with increasing pH; when the pH was higher than pH 4.2, the CL intensity decreased with increasing pH. Thus, pH 4.2 of PNC solution was adopted for further experiment.

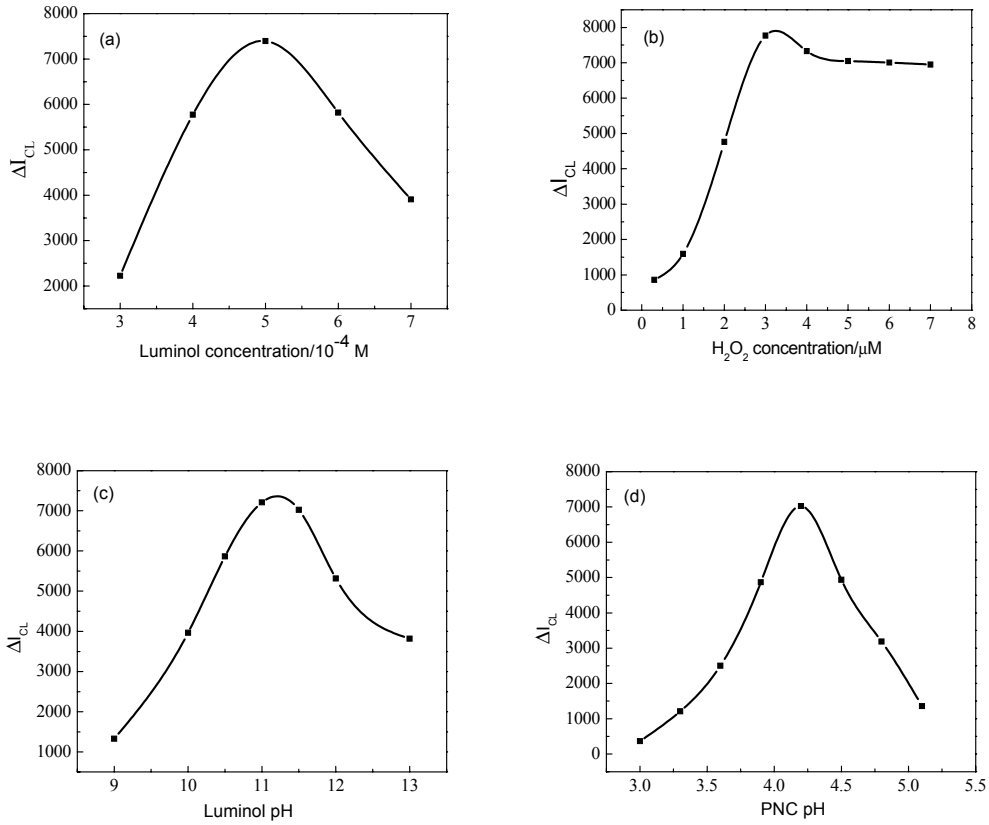



**Fig. S2** Effects of the reactant conditions on the luminol-$H_2O_2$-PNC CL system. (a) Effects of luminol concentration: 0.1 M NaOH-$NaHCO_3$ buffer, 3.0 μM $H_2O_2$, 1.0 μM PNC. (b) Effects of $H_2O_2$ concentration: $5.0\times10^{-4}$ M luminol, 1.0 μM PNC. (c) Effects of luminol pH from 9.0 to 13.0 with the pH of PNC solution at 4.2. (d) Effects of PNC solution pH from 3.0 to 5.1 with the pH of luminol at 11.0. The conditions for c and d are: $5.0\times10^{-4}$ M luminol, 3.0 μM $H_2O_2$, 1.0 μM PNCs.

**Sensitivity for the detection of PNCs.**

Under the optimized conditions, the CL intensity increased linearly with increasing concentration of PNCs in the range of $1.0\times10^{-8}$ M to $1.0\times10^{-6}$ M. The linear regression equation was y=5.2321+72.6862x, with a correlation coefficient of 0.9997.

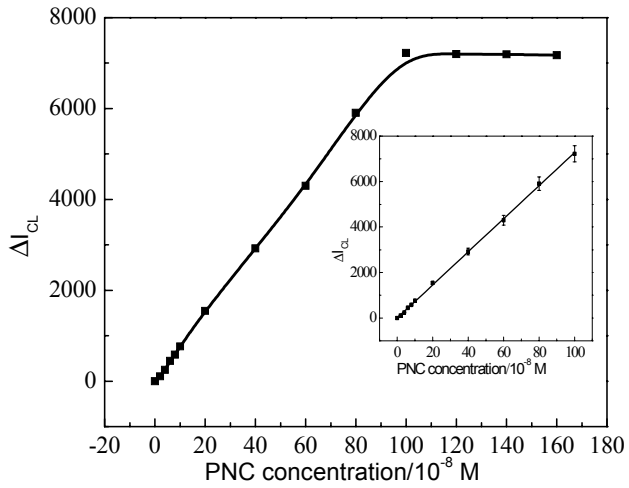



**Fig. S3** The relationship of results for the determination of PNCs. The conditions were as the same as in Fig. S2.

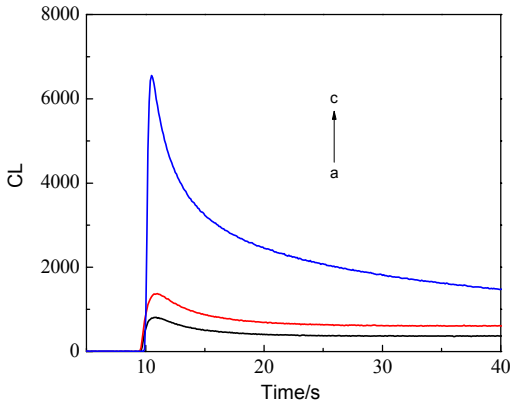



**Fig. S4** Kinetic curves of luminol-$H_2O_2$ chemiluminescence reactions without enhancer (a) and enhanced by $1.0\times10^{-8}$ M AuNPs (b), and PNC modified bio-bar-coded AuNPs (c). Conditions: luminol, $5.0\times10^{-4}$ M; $H_2O_2$, 3.0 μM.

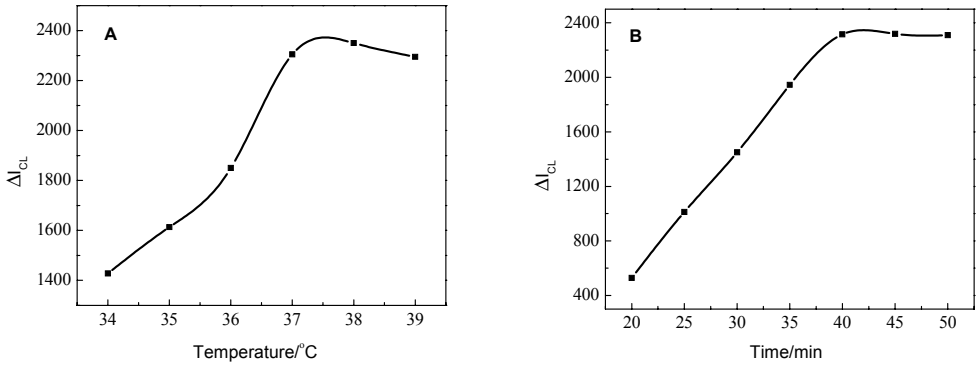
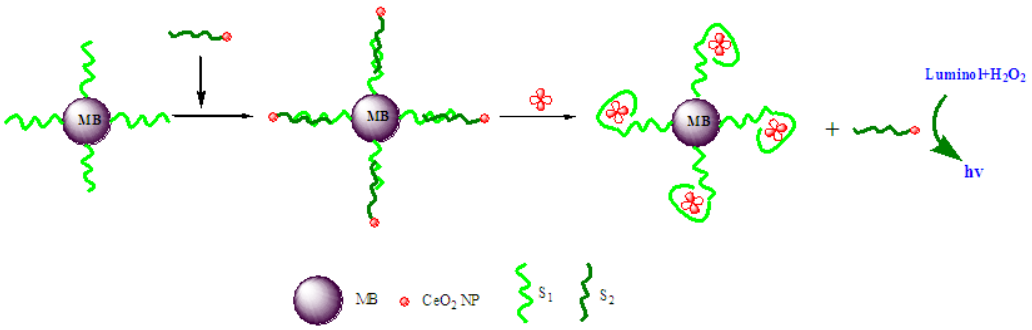



**Fig. S5** Effects of incubation temperature (A) and reaction time (B) of the thrombin and aptamers on the detection of thrombin. The conditions: $5.0\times10^{-4}$ M luminol, $3.0\times10^{-6}$ M $H_2O_2$, $4.0\times10^{-10}$ M thrombin.

**Control experimental with single PNC as labels.**

Control experimental was carried out with single PNCs instead of bio-bar-coded AuNPs used as labels. The detection principle was shown in Scheme S1. The procedure was the same as in the

main text except that 5'-thio-modified $S_2$ was used to label with single PNC. The calibration plot (Fig. S6) exhibited a good linear relationship with the concentration of thrombin ranging from $6.0\times10^{-10}$ M to $1.0\times10^{-8}$ M. The regression equation was y=3.7408x+1.7178, r=0.9996 in which y represents the peak height of CL and C is the concentration of thrombin. A detection limit of $2.8\times10^{-10}$ M thrombin can be estimated using 3σ. A series of eight repetitive measurements of the $1.0\times10^{-9}$ M thrombin solution yielded reproducible CL peaks with relative standard deviation of 7.8%.




**Scheme S1** The schematic illustration of the single PNC catalyzed CL detection of thrombin.

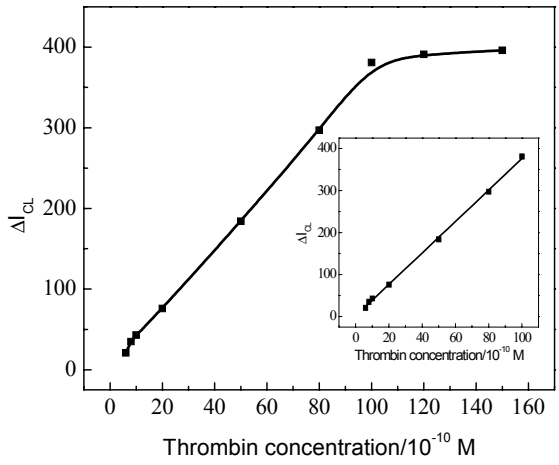



**Fig. S6** The linear relationship between the difference of CL signal and thrombin concentration with PNC as labels.

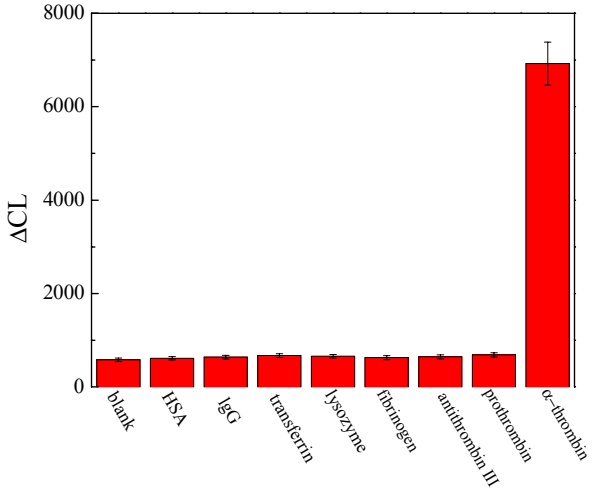



**Fig. S7** Comparison of testing of human α-thrombin and other proteins, showing the specificity of the assay for human α-thrombin (1.0 nM). The concentration of other proteins were 10 nM for HSA, IgG, transferrin, lysozyme, fibrinogen, antithrombin III, and prothrombin.

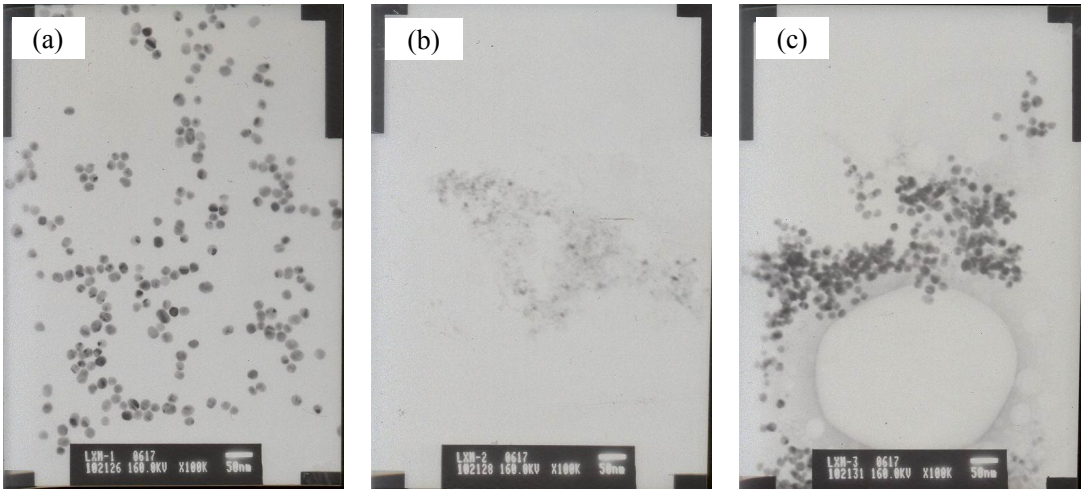



**Fig. S8** TEM images of AuNPs (a), PNCs (b), and AuNPs labeled with PNCs (c).

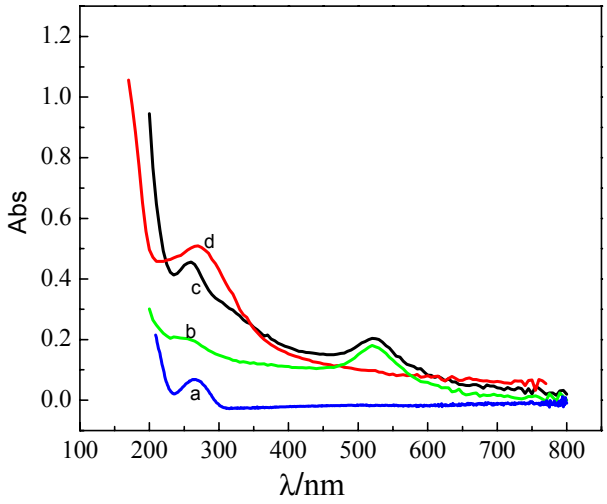



**Fig. S9** UV-vis spectra of DNA (a), AuNPs (b), AuNPs labeled with PNCs (c), PNCs (d).

**Table S1** Effects of quenchers on luminol–$H_2O_2$–PNC CL system.

| **Reaction system** | **CL intensity** |
|---|---|
| Without quencher | 10310 |
| SOD | 928 |
| tert-butanol | 5578 |
| n-butanol | 4908 |
| mannitol | 3432 |

**Table S2** Recovery of Human α-Thrombin spiked into human serum samples.

| Samples | Spiked thrombin ($10^{-11}$ M) | Detected thrombin ($10^{-11}$ M ) | RSD (%, n=5) | Recovery (%, n=5) |
|---|---|---|---|---|
| 1 | 5.0 | 4.7 | 8.2 | 94.0 |
| 2 | 86.0 | 84.3 | 6.3 | 98.0 |
| 3 | 32.0 | 33.8 | 6.8 | 105.6 |
| 4 | 56.3 | 54.2 | 7.2 | 96.3 |
| 5 | 49.2 | 47.0 | 6.4 | 95.5 |
| 6 | 72.9 | 76.3 | 7.4 | 104.6 |
| 7 | 94.3 | 95.4 | 7.7 | 101.2 |

References:

1 J. Liu and Y. Lu, *Nat. Protoc*., 2006, **1**, 246–252.

2 A. Asati, S. Santra, C. Kaittanis, S. Nath and J. M. Perez, *Angew. Chem. Int. Ed*., 2009, **48**, 2308–2312.

3 (*a*) X. Li, J. Xia, W. Li and S. Zhang, *Chem. Asian J*., 2010, **5**, 294–300; (*b*) X. Li, J. Liu and S. Zhang, *Chem. Commun.*, 2010, **46**, 595–597.