Supporting Information

Dual-Targets Fluorescent Nanoprobe for Precise Subtyping of Lung Cancer

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Experimental Section

Materials

Thiol-free ssDNA (Table S1) was purchased from Sangon (Shanghai). Pro-GRP protein was purchased from Tedbio (Wuxi). Chloroauric acid ($HAuCl_4$), trisodium citrate, N-hydroxysuccinimide (NHS), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), selenocysteine were purchased from Sinopharm (Shanghai). Sodium ascorbate was brought from Sigma-Aldrich. Sodium dodecyl sulfate (SDS) was purchased from Sannuo (Shanghai). PBS buffer was brought from Biological Industries. Glutathione (GSH) was purchased from Macklin (Shanghai). Patients' serum was obtained from the Second Hospital of Shandong University). All chemical reagents used in the experiment are of analytical grade and used without any purification. All experimental water is Mill-Q secondary ultrapure water (18.2 MΩ•cm).

Instruments

The transmission electron microscopy (TEM) images of nanoparticles were acquired using a Hitachi HT7700 TEM. Fluorescence and UV-Visible absorption spectra of the probes were measured using Hitachi F-4600 fluorescence spectrophotometer and Shimadzu UV2600 UV-Visible spectrophotometer, respectively. All glassware used in the experiments was pre-treated by soaking in aqua regia (prepared immediately before use by mixing concentrated nitric acid, 68% mass fraction, and concentrated hydrochloric acid, 37% mass fraction, in a 1:3 volume ratio) for 4 h, followed by rinsing with ultrapure water three times and drying in an oven for later use.

Synthesis of Au nanoparticles and the Nanoprobes

Au nanoparticles were prepared using the classical sodium citrate reduction method. Initially, 700 μL of $HAuCl_4$ solution (20 ng/mL) was added to 70 mL of distilled water. The mixture was heated to boiling, and then 3.5 mL of sodium citrate (1 wt%) was

added. The solution was continuously heated and stirred for 20 min, while its color changed rapidly from pale yellow to colorless and eventually to burgundy. After heating, stirring continued until the solution reached room temperature. The prepared Au nanoparticles were stored at 4°C for later use.

The synthesis of 3,3'-diselenodipropionic acid was carried out according to a previously reported method with modifications. The synthesis of selenium-containing ssDNA followed a literature reported with slight modifications. Specifically, 62.6 μL of 3,3'-diselenodipropionic acid solution (0.0011 g in 10 mL) was mixed with 275 μL of NHS (0.0010g in 10 mL) and 275 μL of EDC (0.0016g in 10 mL). After stirring at room temperature for 30 min, 360 μM of selenium-free ssDNA was added to the mixture and stirred at room temperature for 24 h (300 rpm). The reaction mixture was then ultrafiltered to remove byproducts, and the synthesized selenium-containing ssDNA was stored at 4°C. To obtain hybrid double-stranded DNA, equal concentrations of fluorescent-labeled ssDNA and selenium-containing ssDNA were added to PBS buffer (10 mM, pH = 7.4). The mixture was heated to 85°C for 5 min and then cooled to room temperature.

The synthesis of nanoprobes (NPs) involved the addition of 10% SDS surfactant to the prepared Au NPs (3 nM) until the final mass fraction of SDS was 0.1%. After stirring for 30 min at room temperature, 400 nM of DNA-Se hybrid double-stranded DNA was added, and the mixture was exposed to light for 3 h and kept in the dark for 24 h. Subsequently, the solution was centrifuged (13,000 g, 20 min), and the resulting NPs were redispersed in H_2O.

To calculate the DNA loading on the NPs, the optimal excitation and emission wavelengths for FITC-labeled ssDNA (484 nm and 520 nm, respectively) and 5'-TAMRA-labeled ssDNA (553 nm and 585 nm, respectively) were determined. The fluorescence intensities of ssDNA with fluorescence groups at different concentrations (ranging from 20 to 350 nM) were measured to plot standard linear calibration curves. The NPs was diluted to 1 nM in PBS buffer (10 mM, pH = 7.4), and an excess of selenocysteine (10 mM) was added. The mixture was stirred in the dark for 12 h to ensure complete release of the hybrid double-stranded DNA. After centrifugation, the fluorescence intensity of the supernatant was measure *via* fluorescence spectrophotometer. The obtained fluorescence intensity values can be compared with the standard equations to determine the number of DNA strands loaded on the NPs.

The NPs' response under simulated physiological conditions.

Fluorescence recovery of the NPs upon encountering the targets: the NPs were respectively incubated for 1 h with different concentrations of two targets, Pro-GRP (0-150 pg/mL) and Cyfra21-1 (0-300 ng/mL). Fluorescence from FITC and 5'-TAMRA was then collected at 0, 5, 10, 15, 20, 25, 30, 40, 50, and 60 min. Subsequently, the reaction kinetics of the NPs were studied. FITC and 5'-TAMRA fluorescence was immediately collected after adding the targets, Pro-GRP (150 pg/mL) or Cyfra21-1 (300 ng/mL) from the NPs solution (1 nM).

Assess the specificity of the NPs: Pro-GRP (150 pg/mL), Cyfra21-1 (300 ng/mL), and various interfering substances ($MgCl_2$, KCl, PSA, PSMA, CEA, glucose, NSE, Gly, L-His, LgG,Pro-GRP, or Cyfra21-1 at 100 μM) were separately incubated with the NPs

for 1 h. The interferents added for the detection of the target Cyfra21-1 include OC1, TC1, and NC1 (3 μg/mL). Subsequently, the corresponding fluorescence of FITC and 5'-TAMRA was collected for each sample.

To investigate the stability of the NPs at different pH levels and their response to targets under different pH conditions, pH solutions ranging from 6.0 to 8.0 were prepared. The experiment was divided into two groups. In one group, only the NPs solution (1 nM) was added. While in the other group, the NPs solution (1 nM) was supplemented with the target Pro-GRP (150 pg/mL) or Cyfra21-1 (300 ng/mL). These mixtures were co-incubated for 50 min, and then the fluorescence of FITC and 5'-TAMRA was collected.

In order to verify the NPs resistance towards enzymatic cleavage, the NPs were divided into two groups. One group was treated with DNase I (2 U/L), while the other group served as a control. At different time points (10, 20, 30, 40, 50, 60 min), the fluorescence of FITC and 5'-TAMRA was collected for both groups.

To further investigate whether nucleases affect the NPs' response to the target, the NPs were treated with DNase I for 60 min and then divided into two groups. One group was incubated with Pro-GRP or Cyfra21-1 target, while the other group was not treated with the target and served as a control. After 50 min incubation, the fluorescence of FITC and 5'-TAMRA was collected for both groups.

To investigate the effect of glutathione (GSH) concentration on the NPs, 1 nM of the NPs were added to a solution containing 5 mM GSH and incubated at 37°C for 0-12 h. At time points of 0, 1, 2, 4, 8, and 12 h, the samples were centrifuged, and the

fluorescence intensity of the supernatant was measured. Next, to further explore the influence of GSH within 12 h, 1 nM of the NPs were added to solutions containing different concentrations of GSH (0, 10, 50, 100, 200, 500, 1000, and 5000 μM) and incubated at 37°C for 12 h. After centrifugation, the fluorescence intensity of the supernatant was measured.

The investigation of thermodynamic stability involved incubating 1 nM NPs at temperatures of 30, 40, 50, 60, 70, and 80°C for 5 min. After incubation, the samples were centrifuged, and the fluorescence intensity of the supernatant was measured.

Detection Pro-GRP and Cyfra21-1 in serum

To assess the stability of the NPs in serum, the NPs were diluted to 1 nM in serum and incubated at 37 °C for 100 min. The fluorescence at 520 nm and 585 nm was measured at 10-min intervals. The fluorescence intensity ratio at each time interval relative to 0 min was calculated and plotted.

To determine the NPs' response to both targets Pro-GRP and Cyfra21-1 in serum, the NPs were diluted to 1 nM in serum. Firstly, the NPs' response to Pro-GRP was measured. Different concentrations of Pro-GRP protein (20, 50, and 100 pg/mL) were added, and after incubation at 37 °C for 50 min, the fluorescence recovery at 520 nm was measured. The protein concentration was calculated using the response standard curve for Pro-GRP, and the recovery was calculated as follows: Recovery = (Protein concentration calculated from the standard curve / Added protein concentration) * 100. Subsequently, the NPs' response to Cyfra21-1 was measured. Different concentrations of tDNA (50, 100, and 200 ng/mL) were added, and after incubation at 37 °C for 30 min, the

fluorescence recovery at 585 nm was measured. The Cyfra21-1 concentration was calculated using the response standard curve for tDNA, and the recovery was calculated accordingly.

Clinical patients' diagnosis experiment.

To ensure random sampling, serum samples were collected from the Second Hospital of Shandong University. There were 8 samples from NSCLC patients and 6 samples from SCLC patients. The patient's blood was first centrifuged at 4°C for 5 min (4,000 rpm), and the upper-layer serum was collected and stored at -80°C. For SCLC patients, the serum was then diluted ten-fold and added to 1 nM of the NPs, as well as a control group with only serum. After incubation at 37°C for 50 min, the corresponding FITC was collected. Calculate the protein concentration using the response standard curve for Pro-GRP and compare it with the Pro-GRP protein concentration measured by chemiluminescent immunoassay. For NSCLC patients, dilute the NPs to 1 nM in serum, as well as a control group with only serum. After incubation at 37°C for 30 min, the corresponding 5'-TAMRA fluorescence was collected. Calculate the Cyfra21-1 concentration using the response standard curve for the NPs against tDNA and compare it with the Pro-GRP protein concentration measured by chemiluminescent immunoassay.

For the fluorescence response experiment with the NPs in serum from patients with other types of cancer, samples were obtained from five different cancer patients. The serum samples were numbered as follows: 1-5 for gastric cancer, prostate cancer, breast cancer, liver cancer, and ovarian cancer, respectively. The serum was processed in the

same manner as before, added to 1 nM of the NPs and a control group with only serum. After incubation at 37°C for 50 min, the corresponding FITC and 5'-TAMRA fluorescence were collected.

Supplementary Figures

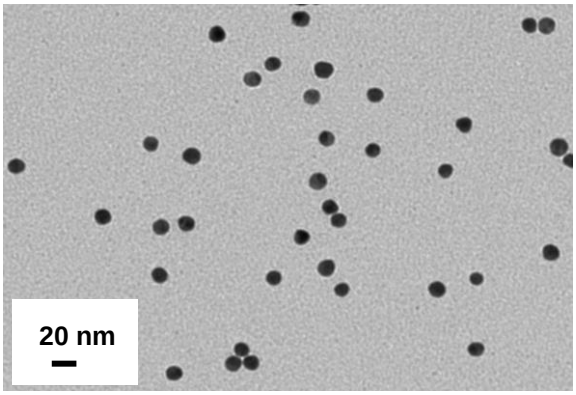

Figure S1. TEM image of the Au nanoparticles.

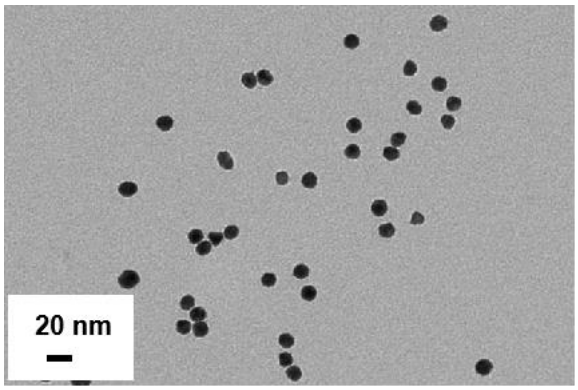

Figure S2. TEM image of the NPs.

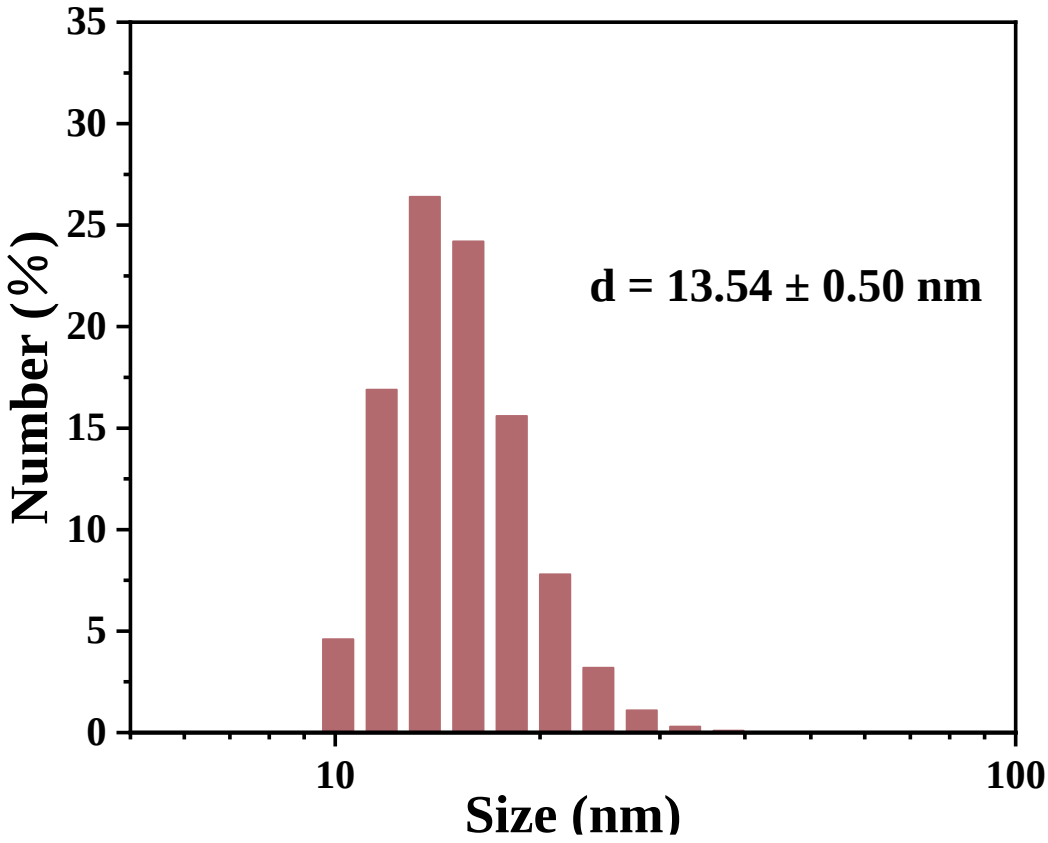

Figure S3. Dynamic light scattering (DLS) data of the Au nanoparticles.

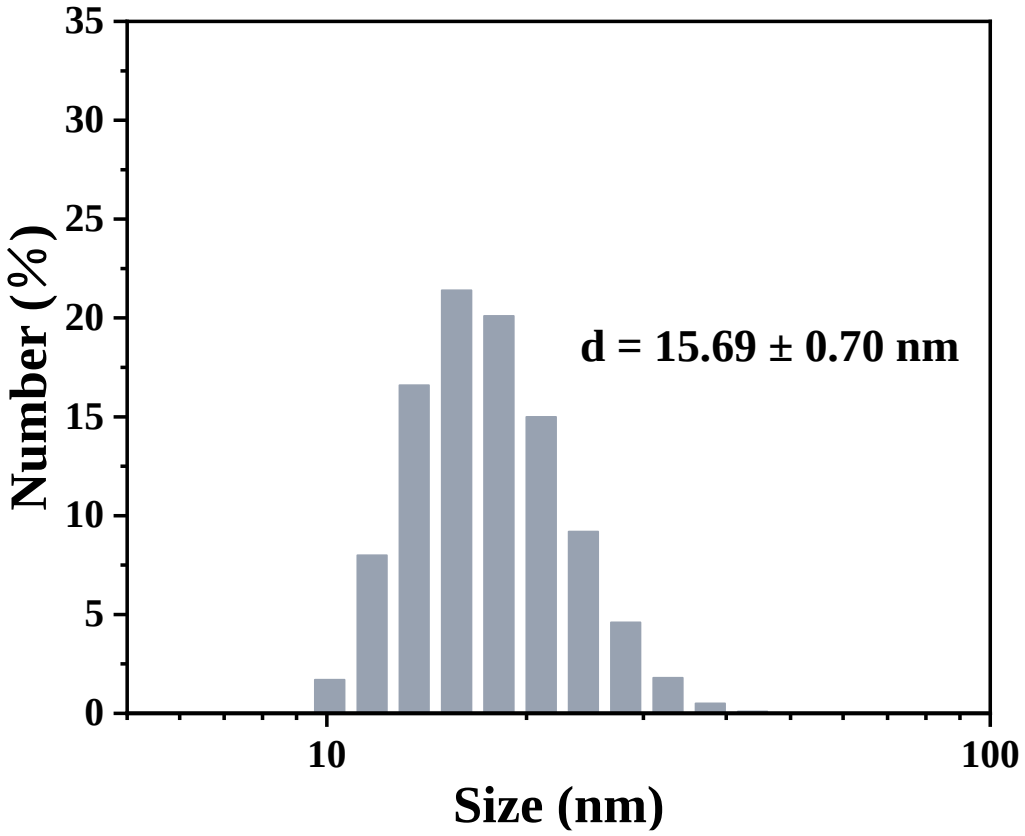

Figure S4. DLS data of the NPs.

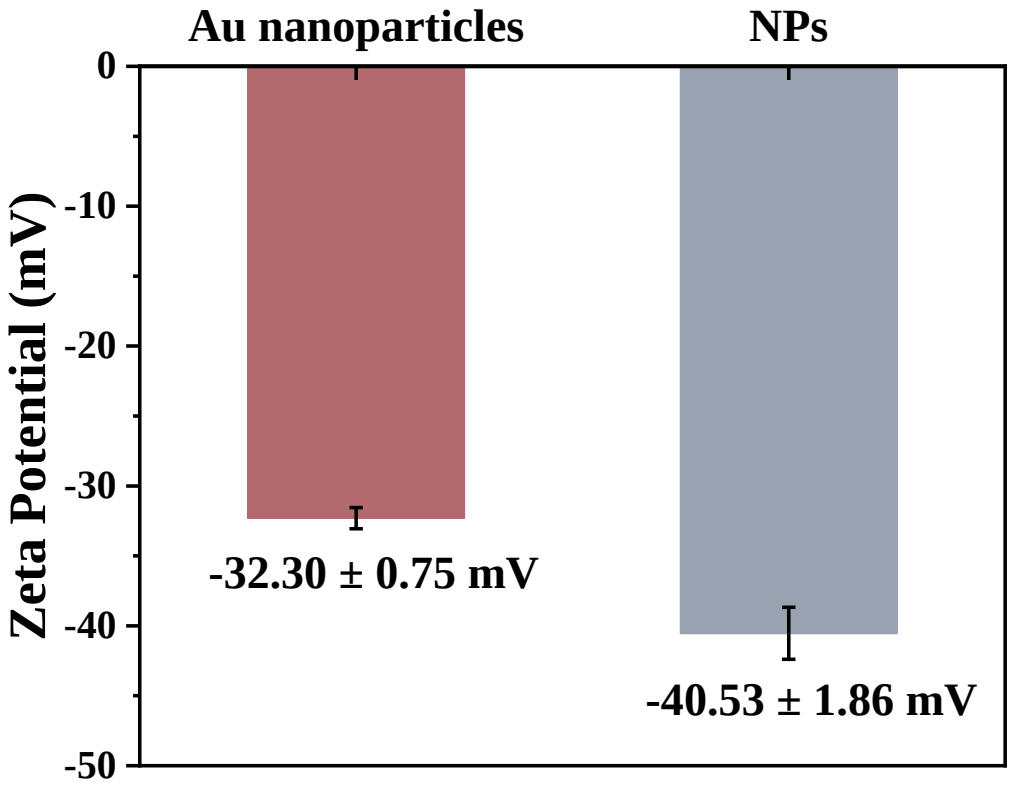

Figure S5. Zeta potential of the (red) Au nanoparticles and (grey) the NPs.

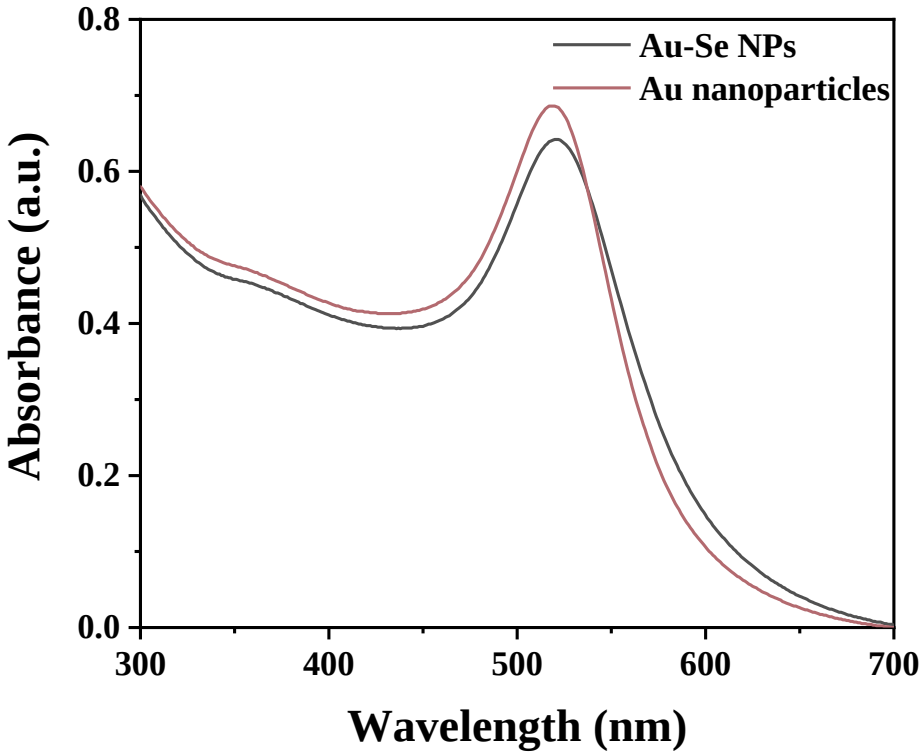

Figure S6. UV-Vis absorption spectra of the (red) Au nanoparticles and (grey) the NPs.

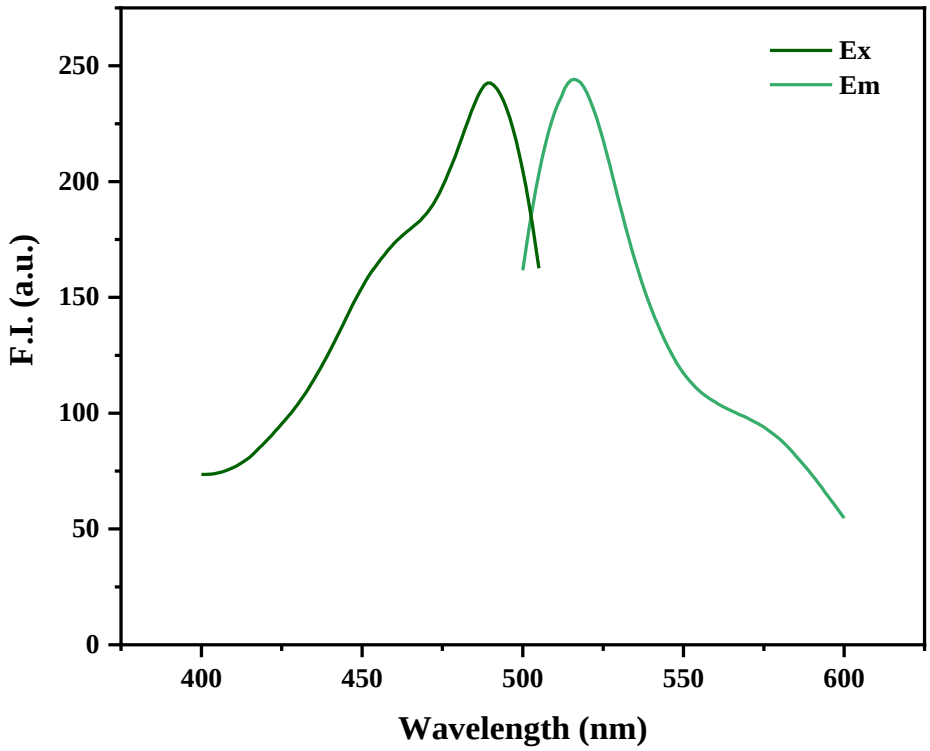

Figure S7. The optimal excitation and emission wavelengths for FITC-labeled ssDNA (Ap).

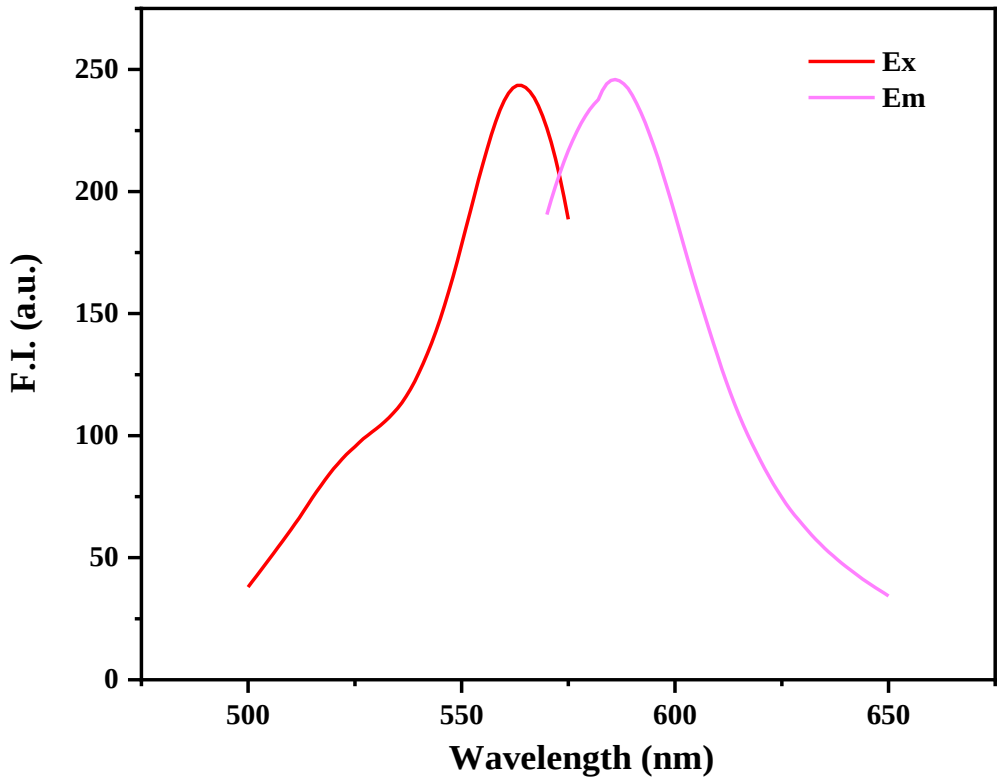

Figure S8. The optimal excitation and emission wavelengths for 5'-TAMRA-labeled ssDNA (P3).

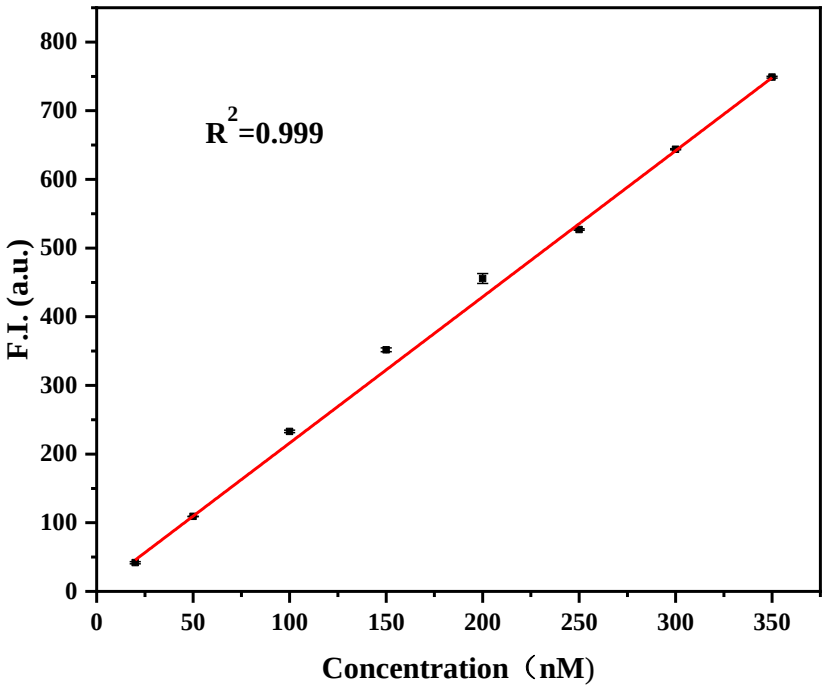

Figure S9. Standard linear calibration curve of FITC-labeled ssDNA (Ap) at different concentrations.

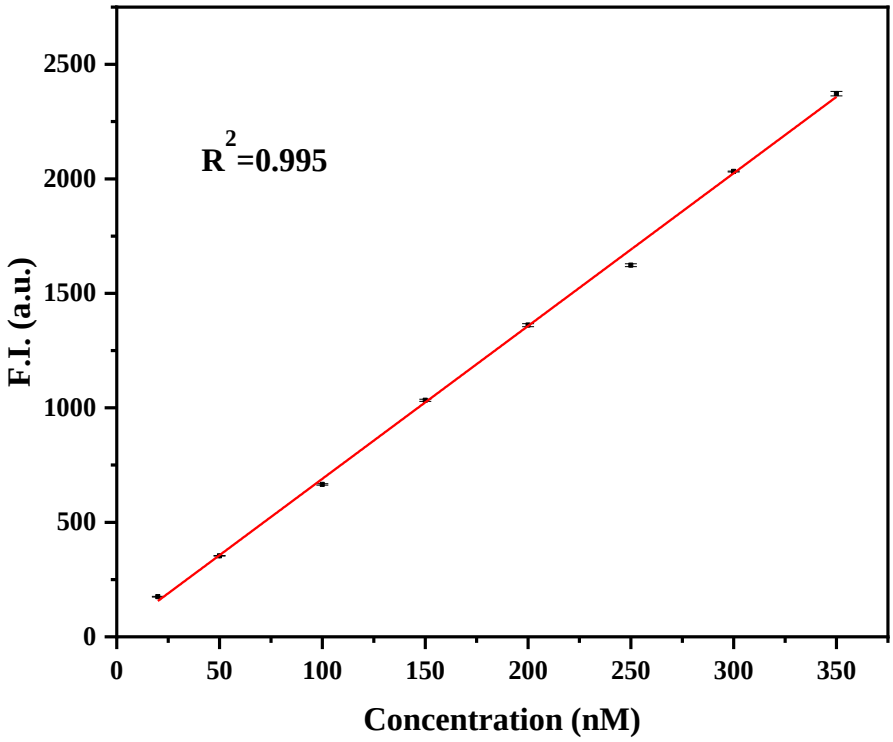

Figure S10. Standard linear calibration curve of 5'TAMRA-labeled ssDNA (P3) at different concentrations.

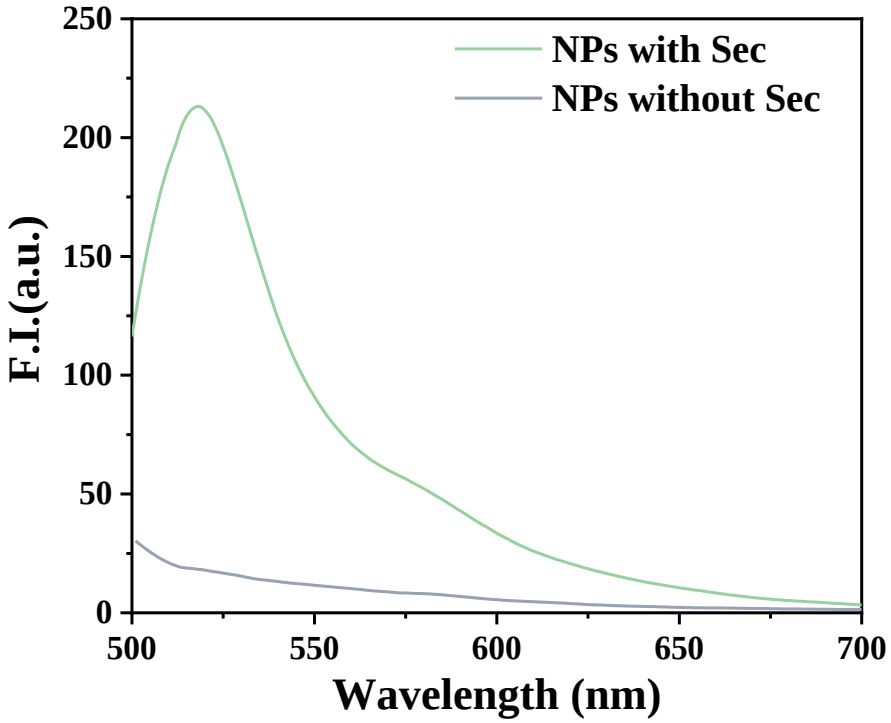

Figure S11. Fluorescence recovery intensity at 520 nm for the NPs in the presence and absence of selenocysteine respectively.

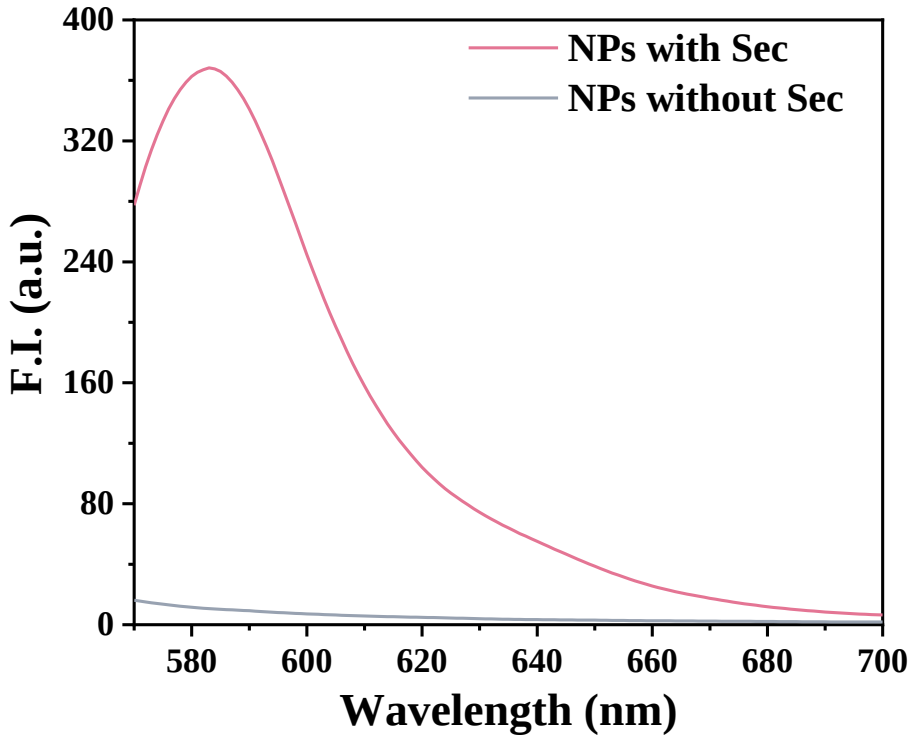

Figure S12. Fluorescence recovery intensity at 585 nm for the NPs in the presence and absence of selenocysteine respectively.

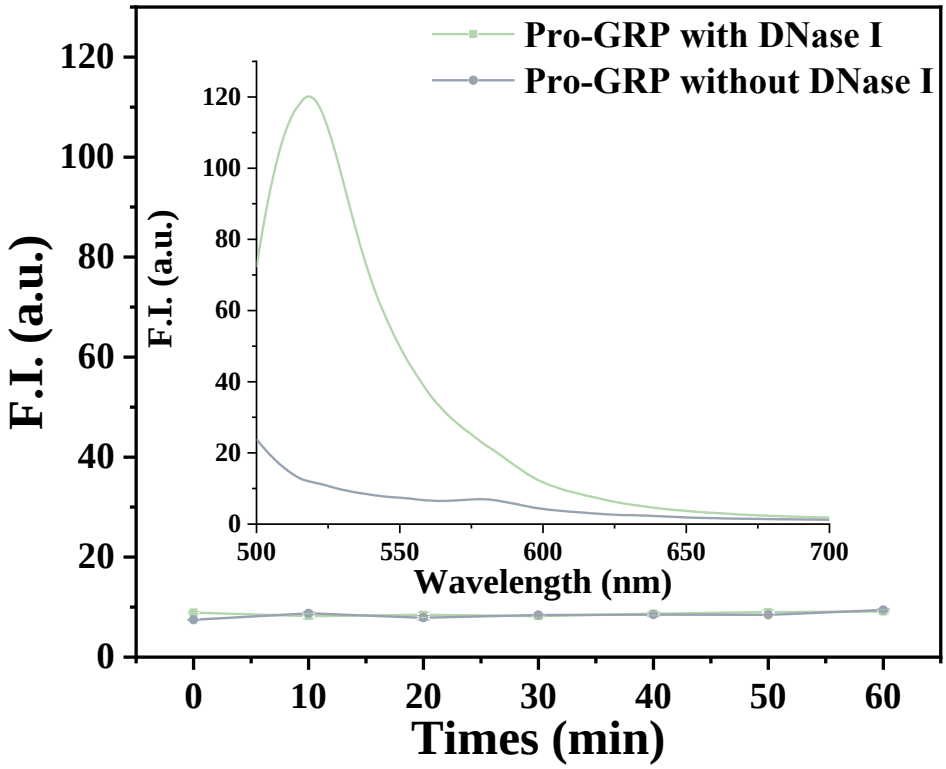

Figure S13. Fluorescence recovery intensity of the NPs at different time points in the presence and absence of DNase I, as well as after the addition of Pro-GRP

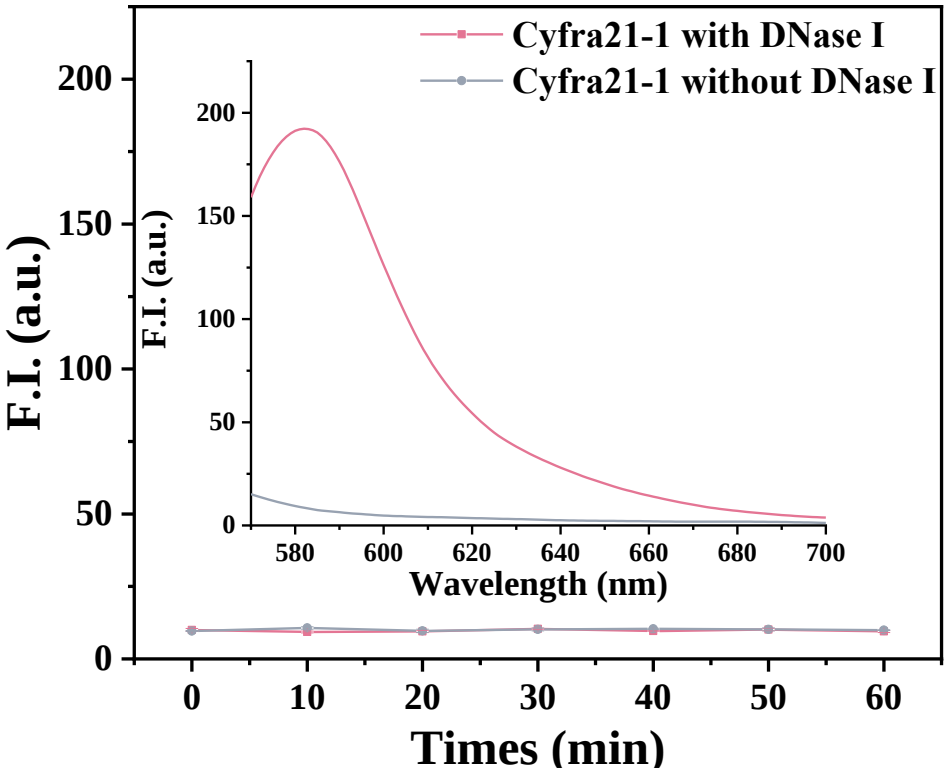

Figure S14. Fluorescence recovery intensity of the NPs at different time points in the presence and absence of DNase I, as well as after the addition of Crfra21-1.

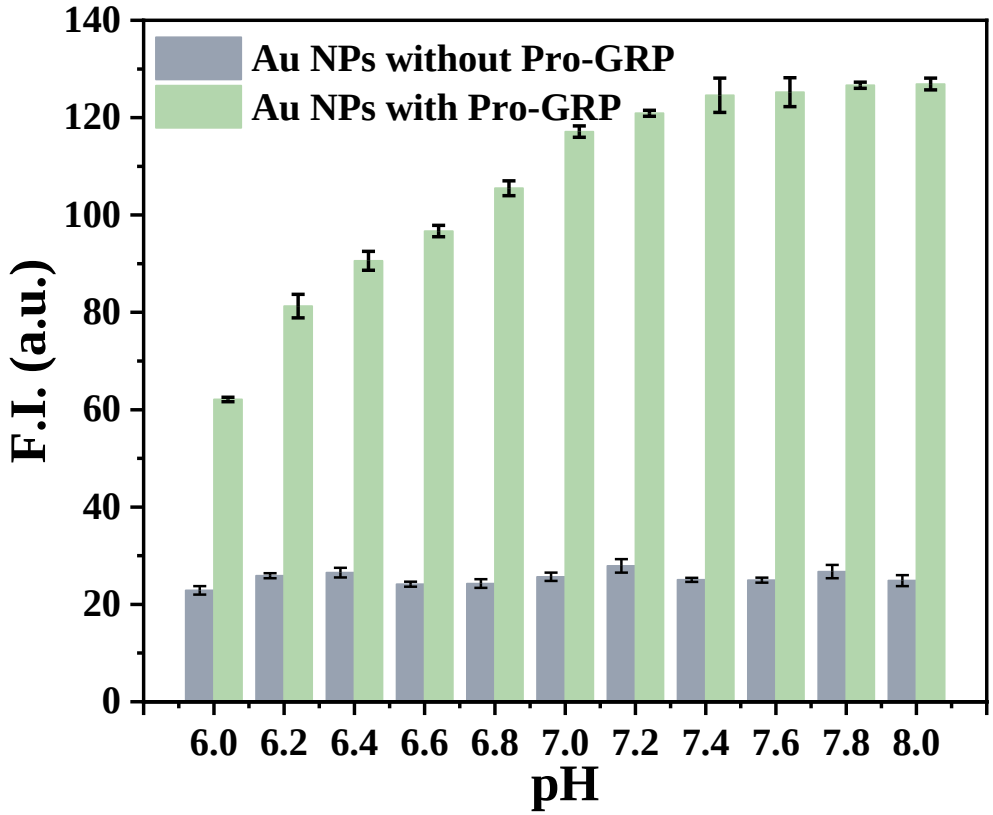

Figure S15. Kinetics response curve of Au-Se NPs to Pro-GRP at different pH levels.

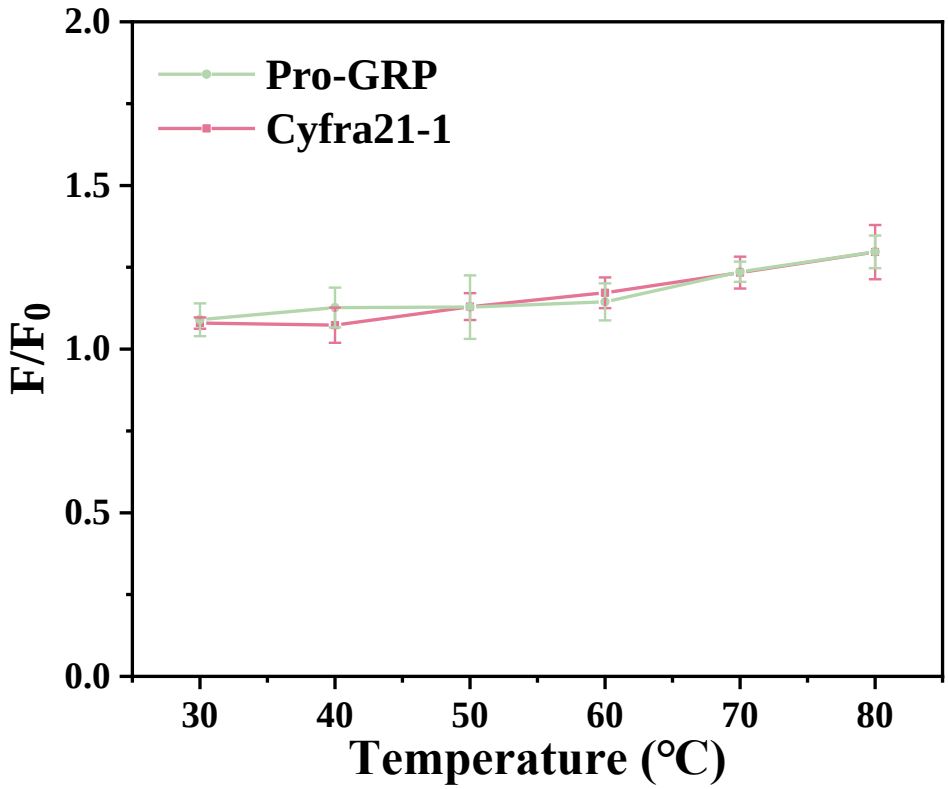

Figure S16. Ratio of fluorescence recovery intensity at 520 nm (green) and 585 nm (red) to initial fluorescence intensity for Au-Se NPs at different temperatures.

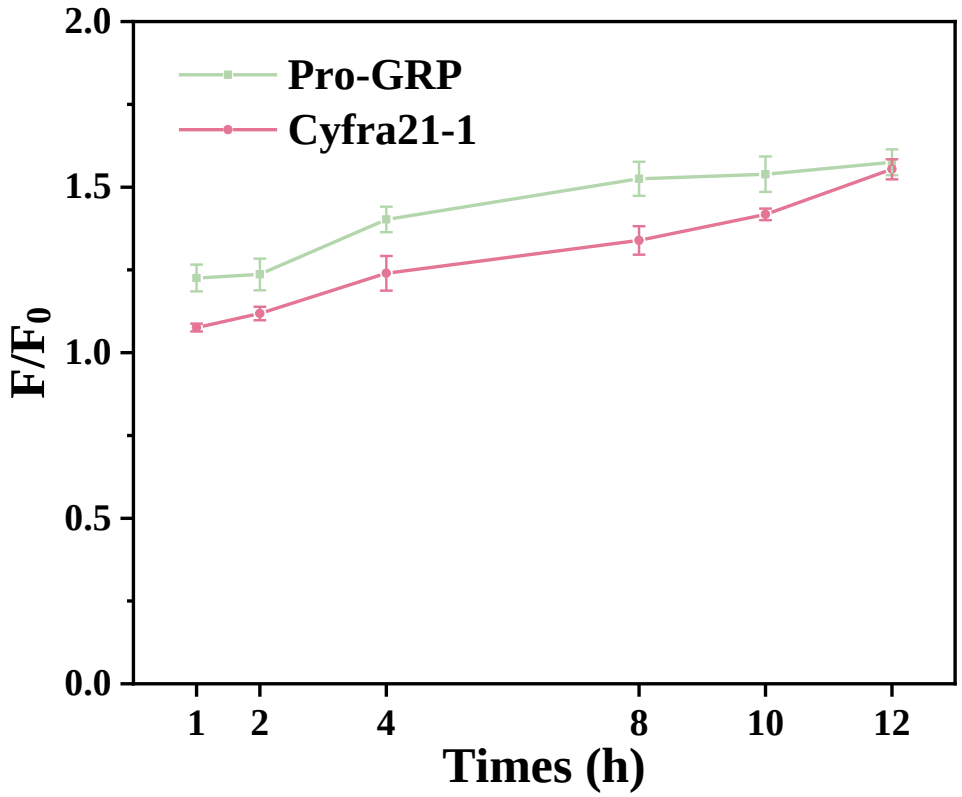

Figure S17. The ratio of fluorescence recovery intensities at (green) 520 nm and (red) 585 nm to initial fluorescence intensities of the NPs under 5 mM GSH for 0-12 h.

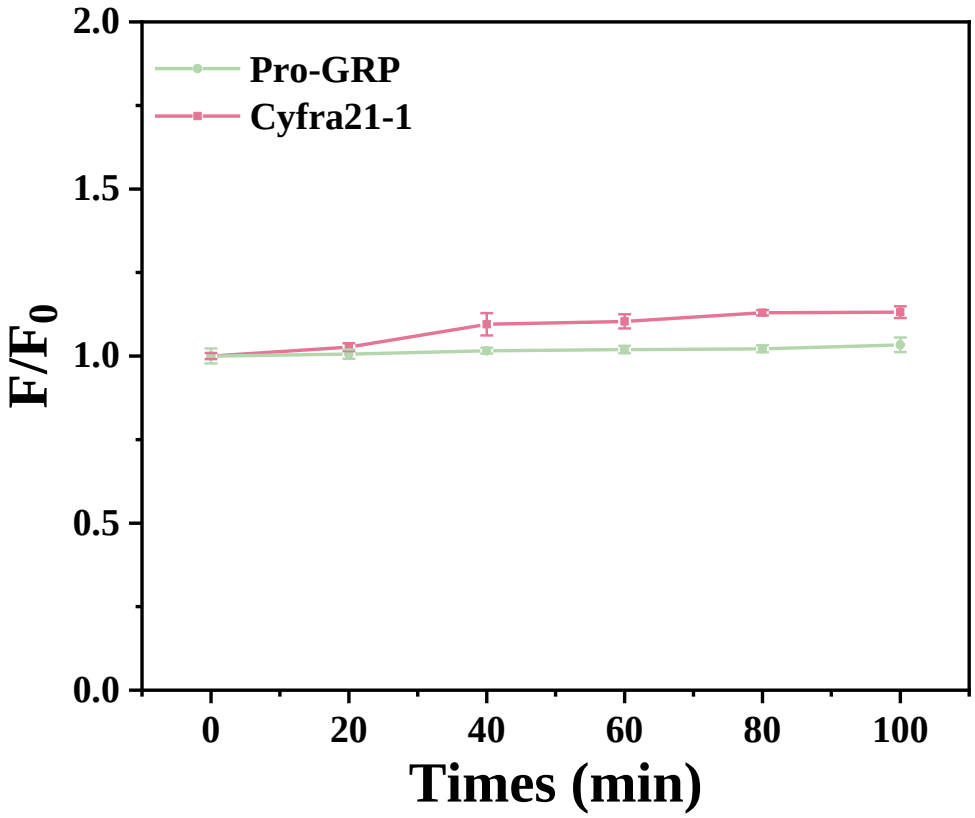

Figure S18. The NPs' stability in serum as prolonged time.

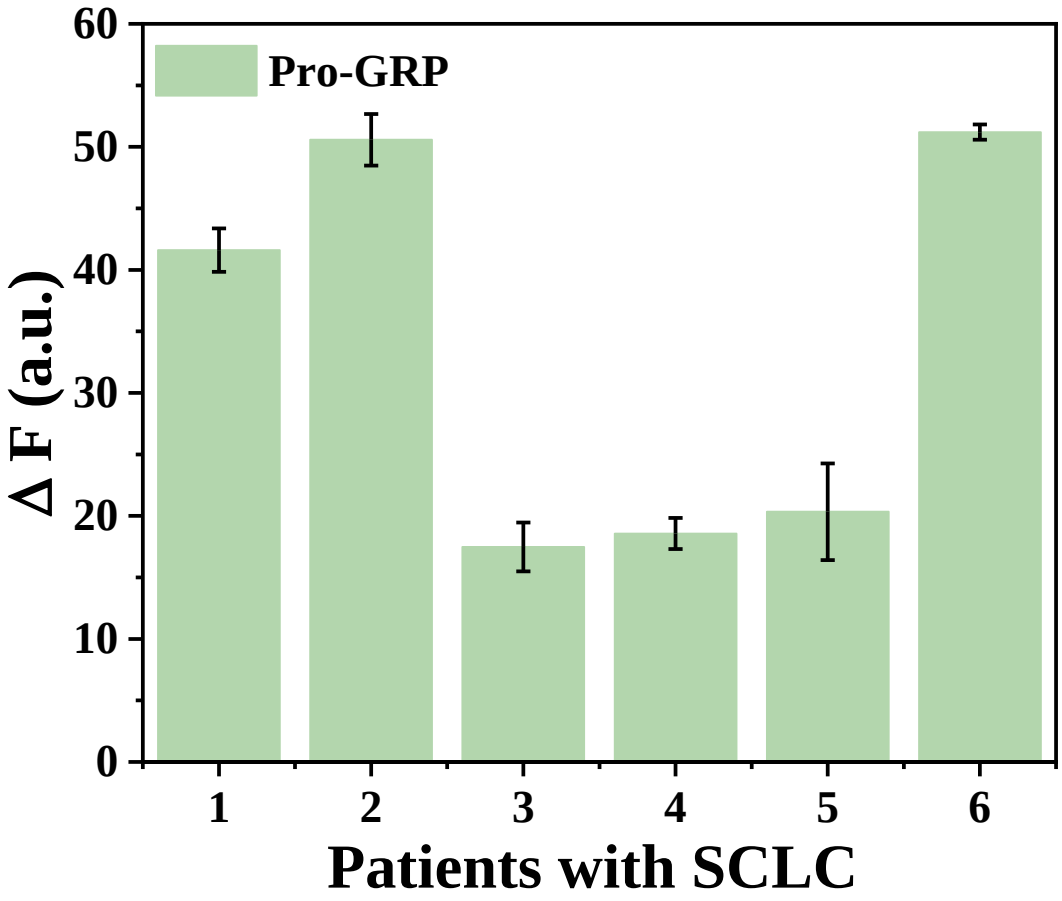

Figure S19. The fluorescence recovery of Au-Se NPs at 520 nm in the serum of SCLC patients.

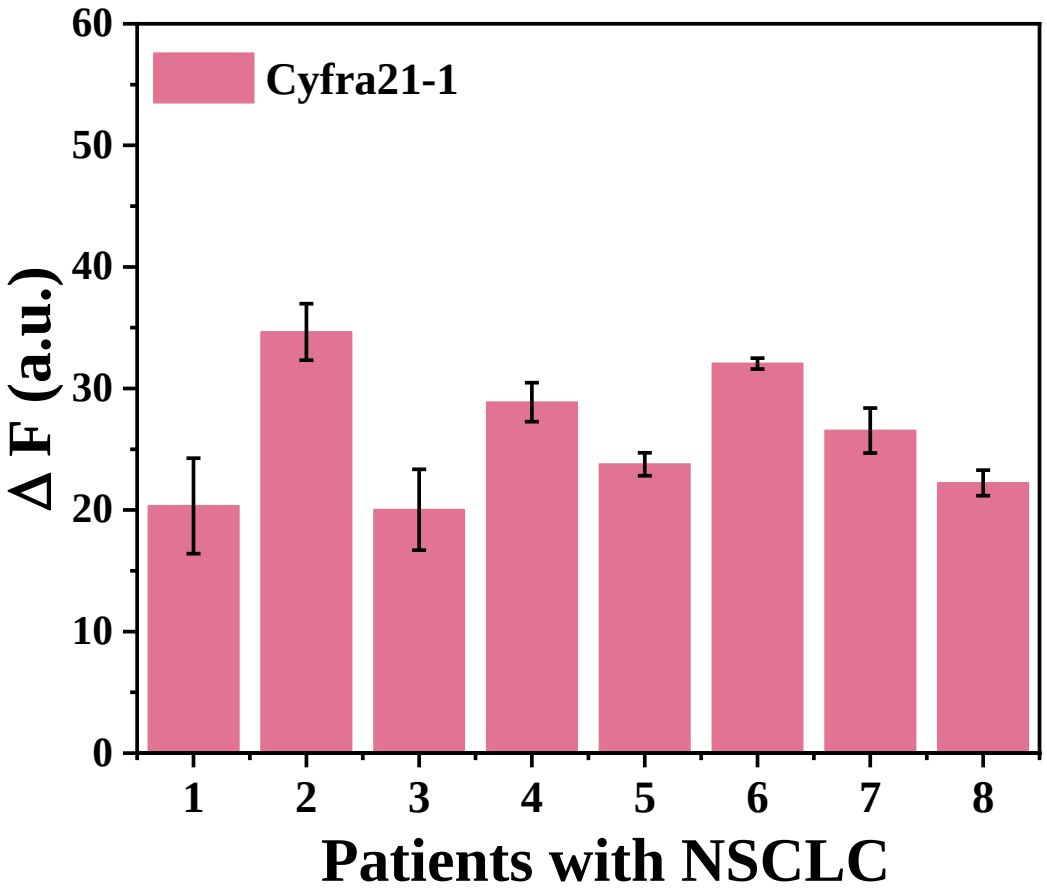

Figure S20. The fluorescence recovery of Au-Se NPs at 585 nm in the serum of NSCLC patients.

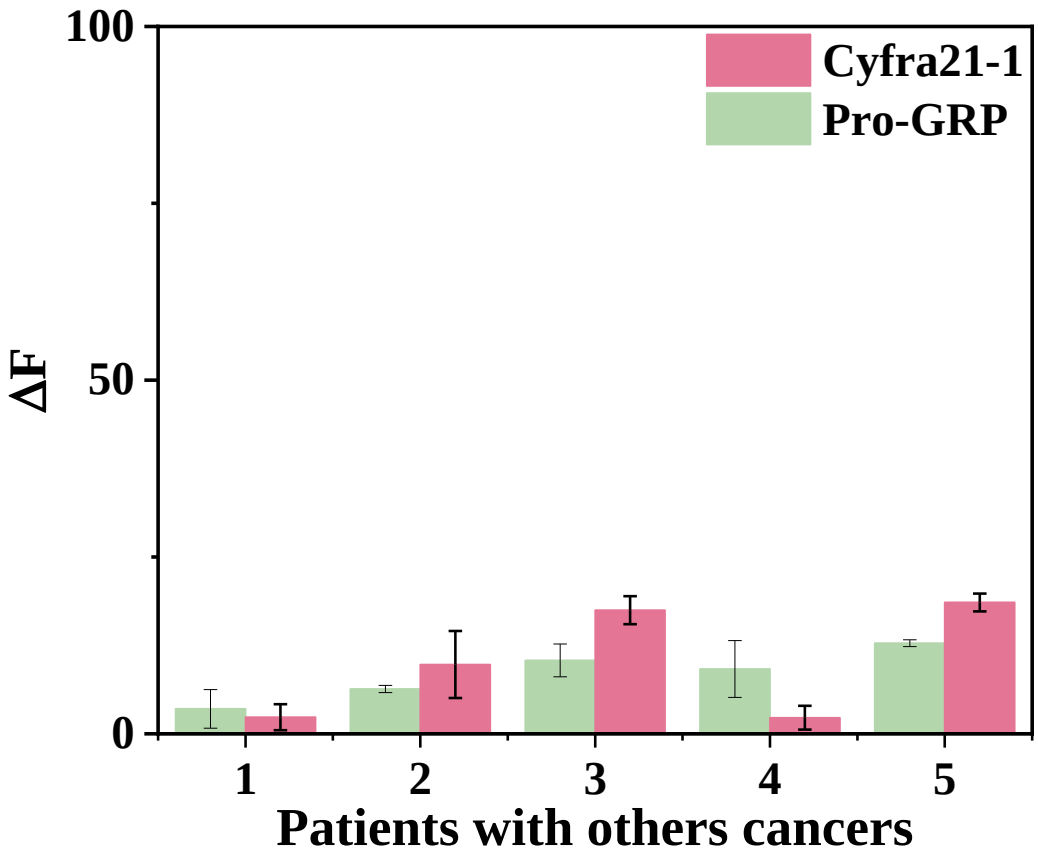

Figure S21. NPs' fluorescence response towards patients with other types of cancer (1: gastric cancer, 2: prostate cancer, 3: breast cancer, 4: liver cancer, 5: ovarian cancer)

Table S1. DNA sequences used in this work.

Oligonucleotide	Sequence(5'-3')
Probe 1 (P1)	NH_2-AAAAAAAAACCTAAGGAAATA
Ampter of Pro-GRP (Ap)	CATGCGGAGTAGATTCGAGCCCAGATAGTCCCTGGTTA TTTCCTTAGG-FTTC
Probe 2 (P2)	5'TAMRA-CGCCCCTGACAC
Complementary Cyfra21-1 (C1)	GAAGGGAGGAATGGTGTCAGGGGCGAAAAAAAAA-NH_2
Cyfra21-1 target (tDNA)	CGCCCCTGACACCATTCCTCCCTTC
One-Base mismatched DNA(OC1)	CGCACCTGACACCATTCCTCCCTTC
Three-Base mismatched DNA(TC1)	CGCCCCGGACACCTTTCCTCCCATC
Non-complementary DNA (NC1)	TTATCCACTCACTTCGGAAGATAGGA

Table S2. The recovery rate of adding Pro-GRP protein to the serum.

Serum samples	Added (pg/mL)	Found (pg/mL)	Recovery (%)	RSD (%)
1	20	19.9	99.5	3.6
2	50	49.2	98.4	1.3
3	100	92.7	96.4	1.2

Table S3. The recovery rate of adding Cyfra21-1 target to the serum.

Serum samples	Added (ng/mL)	Found (ng/mL)	Recovery (%)	RSD (%)
1	50	49.9	99.9	2.7
2	100	97.9	97.9	2.4
3	200	199.9	99.9	0.2