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Two-photon AgNPs/DNA-TPdye Nanosensing Conjugate for Biothiols Probing in Live Cells

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Abstract

In this paper, we present the attempt to fabricate a novel AgNPs/DNA−two-photon dye (TPdye) conjugate as two-photon nanoprobe for biothiols imaging in live cells. The DNA-templated silver nanoparticles can be not only efficient quencher, but also provide a biocompatible nanoplatform for facile delivery of the DNA into living cells. In the presence of biothiols (Cys, Hcy or GSH), the strong interaction between thiol group and silver makes TPdye-labeled single-stranded DNA (ssDNA) to be released from the AgNPs surface, resulting in fluorescence emission of the TPdye recovery, thus realizing the assay of biothiols. Our results reveal that the AgNPs/DNA−TPdye nanosensing conjugate not only is a robust, sensitive, and selective sensor for quantitative detection of biothiols in the complex biological environment but also can be efficiently delivered into live cells and act as a "signal-on" sensor for specific, high-contrast imaging of target biomolecules. Our design provides a methodology model scheme for development of future DNA-templated silver nanoparticles-based two-photon fluorescent probes for in vitro or in vivo determination of biological or biologically relevant species.

Keywords: Two-photon microscopy (TPM), Two-photon absorption (TPA) nanoprobe, DNA-templated silver nanoparticle, Biothiols

Introduction

Biothiols, including thiol-containing amino acids and peptides (cysteine (Cys), homocysteine (Hcy), glutathione (GSH)), as important structural or functional components of many proteins and enzymes play many crucial roles in biological systems.[1] For example, GSH, the most abundant thiol species in the cytoplasm and the major reducing agent in biochemical processes, serve as mediator in many celluar functions including maintenance of intracellular redox activity, xenobiotic metabolism, intracellular signal transduction, and gene regulation.[2] Due to their important roles in physiological activities, great attentions have been paid to the detection of thiol-containing amino acids and peptides.[3] Among these assay protocols for biothiols, fluorescence assays have received much attention for their high sensitivity and great potential in biological imaging. However, the majority of the reported fluorescent methods are mostly based on one-photon excited (OPE) fluorophore,[4] which inevitably produce much larger self-absorption, autofluorescence, and photodamages for biological samples, thus resulting in much limitation of these OPE-based fluorescent strategies in complicated biological samples.[5] Two-photon excitation (TPE) with near infrared (NIR) photons as the excitation source has the advantages of lower tissue autofluorescence and self-absorption, reduced photodamage and photobleaching, high spatial resolution and deeper penetration depth (>500 μm), etc.[6] Together with the development of two-photon microscopy (TPM), TPE has become a powerful tool for research in life science and TP bioimaging applications.[7] Therefore, the TPE-based fluorescent sensing strategies will

have a fascinating prospect for the in vitro or in vivo assay of biothiols in complicated biological conditions.

Although several TPA organic molecular probes have recently been developed and successfully applied in the assay of biothiols,[8] the poor solubility and biocompatibility, slow delivery across membranes and much challenging in design and synthesis for organic molecules, are still the limitations for their wide application in biomedical imaging. Due to the unique properties of oligonucleotide probe, for example facility of design and synthesis, higher structural flexibility and excellent solubility and biocompatibility, the oligonucleotide-based two-photon fluorescent sensing strategy will be an alternate for biothiols assay in biological samples. However, serious limitations of oligonucleotide-based sensing probe for live cell imaging are still present due to the endogenous nuclease degradation, nonspecific binding by DNA/RNA binding proteins, low efficiency of cell penetration and the unquenched high background signal from the probe itself.[9] To address these issues, the noble metal nanocrystals (gold/silver nanoparticles) as the carriers might be a good choice to the fabrication of oligonucleotide-based two-photon fluorescent sensing assembles for biomedical imaging. The noble metal nanoparticles have been proven to be an efficient quencher for fluorescence and these nanocarriers can penetrate into cell easily.[10] In addition, the assembly of DNA sequences on the nanomaterials's surface will improve its ability to resist nuclease degradation.[11]

Considering the excellent properties of noble metal nanocarrier, oligonucleotide-based sensing probe and the TPE-based technique, and as a

continuation of our studies on oligonucleotide/nanostructure conjugate-based sensing designs,[12] we present herein the attempt to fabricate a novel AgNPs/DNA−two-photon dye conjugate as two-photon nanoprobe for biothiols imaging in live cells (Scheme 1). In our proposed strategy, silver nanocrytals were synthesized by using a TPdye-labeled single-stranded DNA (ssDNA) as scaffolds in aqueous solution, that is to say, these AgNPs are stabilized by ssDNA. TPdye-labeled DNA is not only used as DNA template for the formation of AgNPs/DNA conjugate, but also employed as the signal reporter. When the target of biothiol is not presented, the nanoprobe is initially almost nonfluorescent due to the efficient quenching of AgNPs to the adjacent TPdyes. In the presence of biothiols (Cys, Hcy or GSH), the strong interaction between thiol group and silver makes ssDNA to be released from the AgNPs surface, resulting in fluorescence emission of the TPdye recovery, thus realizing the assay of biothiols.

Experimental Section

Chemicals and apparatus

The TPdye (4-[3,6-Bis(1-methyl-4-vinylpyridium iodine)-9H-carbazol-9-yl)] butanoic acid, BMVC-Bu) used in this work was synthesized as described in the Electronic Supplementary Information (ESI), and the TPdye-labeled random ssDNA sequence (DNA−TPdye, 5′-TTTGTTTGTTGGTA-TPdye-3′) was synthesized by TaKaRa Biotechnology Co., Ltd., (Dalian, China). It was dissolved in ultrapure water as stock solutions, and the concentration of oligonucleotide was accurately identified

according to UV absorption at 260 nm. HeLa cells were obtained from the cell bank of Central Laboratory at Xiangya Hospital (Changsha, China). $AgNO_3$ (99.9999%) and $NaBH_4$ were purchased from Sigma-Aldrich. All other chemicals obtained from commercial suppliers were analytical grade and used without further purification. All solutions were prepared using ultrapure water, which was obtained through a Millipore Milli-Q water purification system (Billerica, MA) and had an electric resistance >18.3 MΩ. All experiments were carried out at room temperature.

UV−vis absorption spectra were measured on a Hitachi U-4100 spectrophotometer (Kyoto, Japan). One-photon fluorescence and anisotropy measurements were performed on a PTI QM4 Fluorescence System (Photo Technology International, Birmingham, NJ). Two-photon fluorescence spectra were obtained with a mode-locked Ti:sapphire pulsed laser (Chameleon Ultra II, Coherent Inc.) and then recording with a DCS200PC single photon counting (Beijing Zolix Instruments Co., Ltd.). TPM imaging of HeLa cells were carried out by using an Olympus FV1000-MPE multiphoton laser scanning confocal microscope (Japan). Transmission electron microscopy (TEM) was performed on a JEOL JEM-3010. Energy-dispersive X-ray (EDX) spectra were obtained using the TEM microscope.

Preparation of AgNPs/DNA−TPdye conjugate

Generally, 10 μM DNA−TPdye in 10 mM PBS (pH 7.4) was previously cooled in ice water. Then 300 μL of the 10 μM DNA−TPdye solution was transferred into a 1.5 mL volumetric pipe, and 300 μL $AgNO_3$ stock solution (1.0 mM) was added. The solution was incubated at 0 °C for 15 min to form the DNA/Ag^+ complex. To synthesize

DNA-templated AgNPs, 300 μL freshly prepared $NaBH_4$ solution (1.0 mM) was added into the above obtained solution. After 10 min incubation in ice-water for complete reaction, the AgNPs/DNA−TPdye conjugate was formed and stored at 4 ℃ before use.

***In Vitro* Detection of GSH by the AgNPs/DNA−TPdye Nanosensing Conjugate**.

In a typical assay, an aliquot of 24 μL of the AgNPs/DNA−TPdye conjugate prepared as described in the above was first transferred into a 1.0 mL volumetric pipe and 376 μL of 10 mM PBS (pH 7.4) was added (The final concentration estimated by using DNA−TPdye is 200 nM.), then GSH or other biomolecules was added and incubated at roomtemperature for 10 min. After reaction, the fluorescence spectra of the resulting solutions were recorded. For the OPE measurement, the fluorescence spectra were recorded in a quartz cuvette on PTI QM4 Fluorescence System with the excitation wavelength of 450 nm and the emission wavelengths in the range from 500 to 700 nm with both excitation and emission slits of 10 nm. For the TPE measurement, the two-photon emission fluorescence spectra in the range from 500 to 700 nm were obtained by exciting all samples at 850 nm with a mode-locked Ti:sapphire pulsed laser (Output laser pulses were centered at 850 nm and an average power of 100 mW was as the excitation source. The laser pulses have pulse duration of 120 fs and repetition rate of 80 MHz.), followed by recording with a DCS200PC single photon counting. For the titration experiment, 400 μL of the diluted AgNPs/DNA−TPdye conjugate (The concentration estimated by using DNA−TPdye is 200 nM.) was first brought into a cuvette, and then a continuous titration of GSH was performed by

adding 0.5 μL or 1 μL of 500 μM GSH into the cuvette at each time and 10 min-incubation process was carried out after each addition of GSH. Due to the volume change of the reaction solution upon addition of GSH stock solution was less than 5%, so the volume influence could be ignored.

Cytotoxicity Assay and Live Cell Imaging with the AgNPs/DNA−TPdye Conjugate

HeLa cells were grown in RPMI 1640 medium (Thermo Scientific HyClone) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 100 U/mL penicillin, and 100 U/mL gentamicin at 37 °C in a humidified atmosphere containing 5% CO_2. The cytotoxic effect of AgNPs/DNA−TPdye conjugate was assessed using the MTT assay after treating the cells with different concentrations of the AgNPs/DNA−TPdye conjugate for 24 h in fresh medium (The used mixtures contained 54 μL of the prepared AgNPs/DNA−TPdye conjugate with different concentrations and 946 μL of the fresh cell growth medium.). For cell imaging experiments, cells were seeded in culture plate and grown overnight on glass coverslips at the bottom of the plate. When the cells were ~90% confluent, the coverslips were washed three times with phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). Before incubation with probes, the HeLa cells were incubated with/without 1 mM NMM (*N*-methylmaleimide) for 40min. Then, 1.0 mL of fresh cell growth medium supplemented with 30 μL of the prepared AgNPs/DNA−TPdye conjugate was added to the culture plate. After incubation for 1 h, the cells were washed with Dulbecco's

phosphate buffered saline (DPBS) three times. Two-photon confocal fluorescence imaging of HeLa cells was observed under an Olympus FV1000-MPE multiphoton laser scanning confocal microscope, with a mode-locked titanium-sapphire laser source (120 fs pulse width, 80 MHz repetition rate) set at wavelength 850 nm. Three dimensional images were taken every 2 μm by scanning the samples across a defined section along the z-axis.

Results and discussion

Characterization of the TPdye-labeled DNA

As a newly synthesized fluorescent probe, its optical properties should be carefully examined before biomedical applications. As is exhibited in Figure 1A, this DNA-TPdye displays a strong electronic transition at $\lambda_{max,abs}$ = 460 nm ($\varepsilon = 3.6 \times 10^4$ $M^{-1}cm^{-1}$, ε is the molar extinction coefficient) and its OPE emission spectrum shows a peak maximum at 570 nm with an obvious orange yellow fluorescence under UV lamp excitation. The measurement of fluorescence quantum yield (Φ) with fluorescein as the reference shows that the quantum yield of the DNA-TPdye is 0.16.[13] The fluorescent brightness of this DNA-TPdye (defined as $\varepsilon \times \Phi$) is estimated to be 0.58×10^4 M^{-1} cm^{-1}, suggesting a remarkable signal response capability in bioanalysis. We also measured the two-photon absorption (TPA) action cross section ($\delta \times \Phi$, δ is the TPA cross section) and the TPE emission spectrum of the probe. The maximal TPA action cross section of the probe in aqueous solution was measured to be ca 190 Goeppert-Mayer (GM) at room temperature (λ_{ex} = 850 nm, 1GM = 10^{-50} cm^4 s

photon^{-1} molecule^{-1}) with rhodamine B as the reference.[14] The TPE emission spectrum of the probe is shown in Figure 1B. The sample was excited with femtosecond laser pulses with a central wavelength at 850 nm and a pulse duration of 120 fs. As can be seen, the corresponding maximal emission peak of this TPE emission spectrum is also located at 570 nm and the profile of this TPE emission spectrum matches exactly to that of the OPE emission spectrum, indicating that the fluorescence emission excited at the near-infrared region is purely the two-photoninduced fluorescence.

Measurement in complex biological samples often suffers from the high fluorescence background produced by the ubiquitous endogenous components in the sample matrix,[15] failing the commonly used biosensors without sample pretreatment. The TPE-based fluorescence strategies with NIR photons as the excitation source might be the best choice for the biomedical imaging. To evaluate the performance of this DNA-TPdye in complex conditions, we carried out the fluorescence measurements of this DNA-TPdye in cell media using OPE and TPE techniques. Figure 1C,D shows the OPE and TPE emission spectra of this DNA-TPdye in PBS and the 1640 cell growth media, respectively. One can see from Figure 1C that the cell growth media had a high autofluorescence and dominated the fluorescence spectra from 500 to 650 nm under the OPE. Furthermore, the fluorescence emission intensity inversely increased when the DNA-TPdye was dispersed into the cell growth media, which might be a result from the high autofluorescence of the cell growth media. However, in contrast to the OPE measurements, one can find that the TPE

fluorescence emission intensity and its emission spectrum in the cell growth media were almost the same as in the PBS (Figure 1D). These results suggest that this DNA-TPdye is a promising fluorescence emitter for TP imaging applications in complex biological samples and the TPE method is more suitable for biological assays compared with the OPE-based methods, owing to the low background fluorescence.

Formation and Characterization of the AgNPs/DNA-TPdye Conjugate

Because ssDNA sequences have been proven to be used as the template for synthesis of silver nanoparticles (AgNPs),[16] the nanosensing conjugate of AgNPs/DNA-TPdye was prepared by using a TPdye-labeled ssDNA with 14-bases length (DNA-TPdye) as the template via reducing the DNA-TPdye/Ag^+ complex with $NaBH_4$. We can see from Figure 2A that the fluorescence intensity of the DNA−TPdye solution decreases proportionally with the increasing amounts of Ag^+ and at the same time the concomitant absorption peak of AgNPs centered at around 410 nm can be observed (Figure 2C), indicating the growing number of DNA−TPdye formed the AgNPs/DNA-TPdye conjugate, thus resulting in the quenching of the TPdye's fluorescence by way of energy or electron transfer processes.[17] When the concentration of Ag^+ added reached about 16 μM, the fluorescence of 200 nM DNA−TPdye was nearly completely quenched. We can also observe from Figure 2C that the absorption peak at 410 for curve c is different from curve b, the reason might be that the AgNPs/DNA conjugate was destroyed and the AgNPs aggregated when GSH was added, so the absorption intensity at 410 nm is decreased and red shift. It is

worth to note that for the non-template-synthesized AgNPs/DNA-TPdye conjugate prepared by directly mixing AgNPs with DNA-TPdye, the maximal quenching efficiency is only about 35% (Figure 2D), much lower than that of template-synthesized AgNPs/DNA-TPdye conjugate under the same condition. So the template-synthesized strategy for the fabrication of AgNPs/DNA-TPdye nanosensing conjugate is the best choice. The AgNPs/DNA−TPdye conjugate was then further characterized by transmission electron microscopy (TEM) and energy-dispersive X-ray (EDX) spectroscopy to obtain its morphological profile and structural information. We can see from Figure S1A (ESI) that the AgNPs/DNA−TPdye conjugate shows uniform and monodispersed sphere shapes with a diameter distribution from 3.0 to 8.0 nm. EDX analysis of the AgNPs/DNA−TPdye conjugate shows the coexistence of Ag, O, N and P elements which belongs to AgNPs and oligonucleotides (Figure S1B, ESI), respectively, further indicating that the coverage of DNA−TPdye on the AgNPs surface and the successful formation of AgNPs/DNA−TPdye nanosensing conjugate.

Validation of the Sensing Scheme

Scheme 1 illustrates the signaling mechanism of the AgNPs/DNA−TPdye nanosensing conjugate. To verify this design scheme, we investigated the real-time records of OPE fluorescence intensity and the anisotropy changes of DNA−TPdye in the PBS solution upon formation of AgNPs and subsequently addition of GSH (Figure 3A). In aqueous solution, DNA−TPdye has a strong fluorescence emission, while the fluorescence emission was quenched greatly after the formation of AgNPs

(curve a), indicating the interaction of TPdye with the formed AgNPs, allowing the energy transfer process to occur. Distinct increase of fluorescence emission after GSH addition verified the feasibility of the sensing scheme. Curve b shows the fluorescence anisotropy (FA) value change of the DNA−TPdye under the same conditions. The FA value of DNA−TPdye at the free state in the buffer is low (0.06). However, it underwent an enhancement (0.12) upon formation of AgNPs. This result indicates that the DNA-templated synthesis of AgNPs created a larger mass complex, which hindered the rotation diffusion rate of the labeled TPdye.[18] The significant difference in the FA values of DNA−TPdye and the AgNPs/DNA−TPdye further confirms the formation of AgNPs/DNA−TPdye conjugate. The FA value was reduced from 0.12 to 0.10 with addition of GSH, meaning that the strong competitive assembly of GSH on the AgNPs surface freed the DNA−TPdye from the AgNPs surface. It is worth noting that the fluorescence intensity and anisotropy changes of AgNPs/DNA−TPdye nanosensing conjugate reached equilibrium within a few minutes. This indicates the potential of our assay for rapid and real time monitoring of the target in homogeneous solutions. Further inspection of the AgNPs/DNA−TPdye nanosensing conjugate was also performed by using gel electrophoresis (Figure 3B). The results show that there is no significant DNA−TPdye band on the gel image for the supernatant of AgNPs/DNA−TPdye conjugates (lane 2), while for the supernatant of AgNPs/DNA−TPdye conjugates with addition of GSH, there is a clear DNA−TPdye band on the gel image (lane 3), indicating that the DNA−TPdye fell off from the AgNPs surface due to the competitive assembly of GSH and stayed in the

supernatant after centrifugation. These results agree well with the above fluorescence emission experiments.

AgNPs/DNA−TPdye Nanosensing Conjugate for Biothiols Assay in Buffer Solution

To evidence the potential application of the AgNPs/DNA−TPdye conjugate as a high-performance fluorescent probe for detection of thiol–containing amino acids and peptides, this sensing conjugate was titrated with varying concentrations of biothiols (Here, GSH was chosen as the model). Figure 4A shows the fluorescence spectra changes of the sensing conjugate in the presence of different concentrations of GSH. The fluorescence emission intensity at 570 nm was significantly increased with an increase in the GSH concentration. When the concentration of GSH was increased to 15 μM, no significant fluorescence change can be observed, indicating that the sensing fluorescence response of the nanosensing system saturates at 15microM of GSH under the present 200 nM of DNA−TPdye as the nanoconjugate's substrate, and the detection limit that is taken to be three times the standard derivation in blank solution is ca 42 nM.

To evaluate the selectivity of our assay, the TPdye fluorescence intensity changes with the addition of different targets (GSH, Cys, Hcy, and other amino acids) were studied. As shown in Figure 4B, when GSH/Cys/Hcy was presented, the system gives a significant TPdye fluorescence enhancement, while clear increase in fluorescence emission can not be observed upon addition of other amino acids under the same conditions. The binding of GSH/Cys/Hcy to AgNPs is highly selective due

to the high values of the formation constants for the S-Ag bond which lead to an assay with high specificity.

AgNPs/DNA-TPdye Nanosensing Conjugate for Biothiols Probing in Live Cells

Finally, we investigated the capabilities of the AgNPs/DNA−TPdye conjugate as a fluorescent sensor for live-cell imaging of biothiols. Before application of the sensing assemble in cell imaging, the cytotoxicities of AgNPs/DNA−TPdye conjugate on HeLa cells were evaluated using the standard cell viability assay, the MTT assay. One can see from Figure S2 (ESI) that much high cell viability was observed (survival rate was higher than 85% in 1.0×10^4 cells/well) even after the HeLa cells were treated with AgNPs/DNA−TPdye conjugate concentrations up to 180 nM for 24 h (The concentration of the AgNPs/DNA−TPdye conjugate refers to the concentration of DNA-TPdye). The results show that no significant influence on HeLa cells can be observed under a certain amount of AgNPs/DNA−TPdye conjugates (for example 180 nM) and a certain incubation time (for example 24 h). The TPE confocal fluorescence imaging in HeLa cells as the model was then carried out. HeLa cells were incubated with AgNPs/DNA−TPdye conjugate or DNA−TPdye in buffer solution for 1 h at 37 ^{0}C, and after washing three times with PBS the fluorescence images were obtained using scanning confocal microscopy. As shown in Figure 5, a significant TPE fluorescence emission in the fluorescence image was observed for the cells incubated with the AgNPs/DNA−TPdye conjugate while clear fluorescence signals can not be observed for the cells incubated with DNA−TPdye, indicating that AgNPs/DNA−TPdye conjugate efficiently penetrated cell membranes and successful

GSH measurement in live cells. As a control, if the NMM (N-methylmaleimide) was employed as a thiol-blocking reagent in a parallel experiment, no appreciable contrast in the fluorescence images can be observed. To further verify the internalization of AgNPs/DNA−TPdye conjugate in HeLa cells, Z-scanning confocal imaging was performed (Figure S3, ESI). It was clear that bright fluorescence was present throughout the whole cells, which suggested efficient delivery of the AgNPs/DNA−TPdye conjugate in the cytosol. This revealed that the proposed AgNPs/DNA−TPdye sensing conjugate can be successfully used for the live-cell imaging of biothiols.

Conclusion

In summary, we have developed a sensitive and selective two photon nanoprobe for live-cell imaging of biological thiols via the formation of DNA-templated AgNPs. The conjugation of DNA with silver nanoparticles by DNA-templated synthesis is simple without needing either surface functionalization of the nanoparticles or covalent labeling DNA. Most importantly, introduction of a two photon dye as the signal reporter effectively eliminates the self absorption and autofluorescence of the biological molecules in biological matrixes due to long-wavelength excitation and offers high resolution, larger depth penetration, as well as less photodamage. TP confocal fluorescence microscopy experiments with HeLa cells suggested that the AgNPs/DNA−TPdye conjugate was successfully delivered into live cell and acted as an efficient sensor for specific, high-contrast imaging of biothiols. We envision that

this new AgNPs/DNA−TPdye conjugate-based strategy for developing robust biomolecules sensors is expected to hold great potential for in vitro and in vivo applications in medical research and clinical diagnostics.

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Electronic Supplementary Information: Additional information as noted in text.

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Figure Captions

Scheme 1 Schematic illustration of the fluorescent detection for biothiols by using the AgNPs/DNA-TPdye conjugate. When the target of biothiols is not presented, the nanoprobe is initially almost nonfluorescent due to the efficient quenching of AgNPs to the adjacent TPdyes. In the presence of biothiol (for example GSH), the strong interaction between thiol group and silver makes ssDNA to be released from the AgNPs surface, resulting in fluorescence emission of the TPdye recovery, thus realizing the assay of biothiols.

Fig. 1 (A) Absorption (dashed lines) and one-photon emission spectra (solid lines) of 1 μM DNA-TPdye in PBS solution (pH7.4). (B) The TPE emission spectrum of the DNA-TPdye (200 nM) in PBS. OPE (C) and TPE (D) emission spectra of the only cell growth media (curve a), DNA−TPdye (200 nM) in PBS solution (curve b), and DNA−TPdye (200 nM) in cell growth media (curve c). For the OPE measurement, the fluorescence spectra were recorded in a quartz cuvette on PTI QM4 Fluorescence System with the excitation wavelength of 450 nm and the emission wavelengths in the range from 500 to 700 nm with both excitation and emission slits of 10 nm. For the TPE measurement, the two-photon emission fluorescence spectra in the range from 500 to 700 nm were obtained by exciting all samples at 850 nm with a mode-locked Ti:sapphire pulsed laser (Output laser pulses were centered at 850 nm and an average power of 100 mW was as the excitation source. The laser pulses have pulse duration of 120 fs and repetition rate of 80 MHz.), followed by recording with a DCS200PC

single photon counting.

Fig. 2 (A) Fluorescence emission spectra of DNA-TPdye under the different concentrations of Ag^+ (From up to bottom: 0, 0.5, 1, 4, 8, 12，16 and 20 μM, respectively), the arrow indicates signal changes as the Ag^+ concentrations increase. The concentration of DNA-TPdye is 200 nM and the used $NaBH_4$ concentration is same as Ag^+. The experiment was carried out as described in experimental section. Inset: Quenching efficiency of the fluorescence emission at 570 nm (QE%) of the DNA-TPdye with various concentrations of Ag^+. λ_{ex} = 450 nm. (B) Fluorescence emission spectra of DNA-TPdye under different experimental conditions: 'curve a', 200 nM DNA-TPdye + 20 μM Ag^+ in PBS; 'curve b', 'a' + 20 μM $NaBH_4$; 'curve c', 'b' + 12.5 μM GSH; λ_{ex} = 450 nm. (C) The corresponding UV-vis absorption spectra of (B), showing formation of the DNA-templated AgNPs. (D) Effect of different concentrations of AgNPs colloid solution on the fluorescence emission of DNA-TPdye (200 nM). The arrow indicates signal changes as the AgNPs concentrations increase. Inset: Quenching efficiency of the fluorescence emission at 570 nm (QE%) of the DNA-TPdye with various concentrations of AgNPs. The experimental process is: 300 μL freshly prepared $NaBH_4$ solution (1.0 mM) was added into the 600 μL of $AgNO_3$ solution (0.5 mM) and 10min-incubation in ice-water for complete reaction was carried out. Then DNA−TPdye stock solution was added into the above obtained AgNPs with different concentrations. The final concentration of DNA-TPdye is 200 nM. Note: The concentration of AgNPs refers to

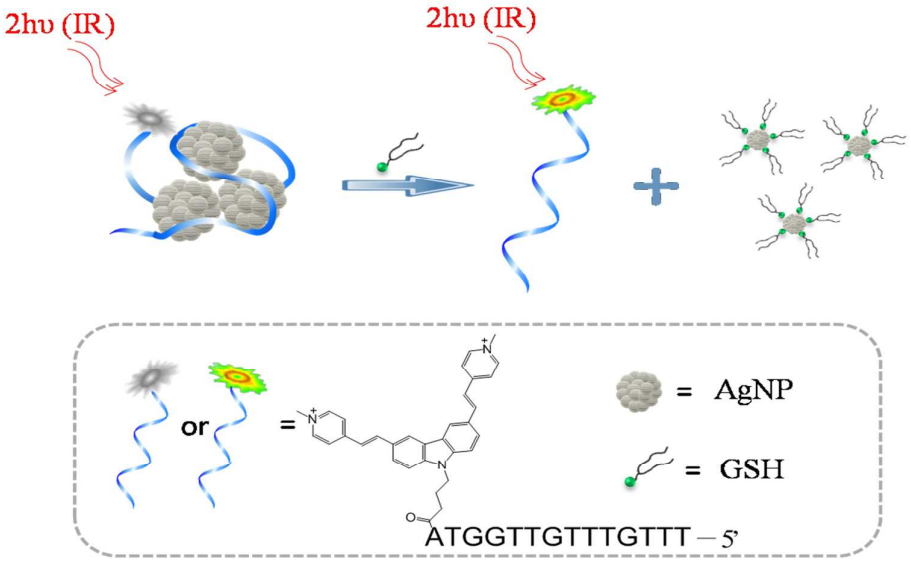
the concentration of Ag^+.

Fig. 3 (A) Real-time fluorescence records (curve a) and fluorescence polarization changes (curve b) of DNA-TPdye (200 nM) upon the formation of AgNPs/DNA-TPdye conjugate and addition of GSH. The transitions between each regime are marked with an arrow. Experimental process: 400 μL of the 200 nM DNA−TPdye in 10 mM PBS (pH 7.4) was first transferred into a quartz cuvette. After about 3 min under stirring, 8 μL of 1.0 mM $AgNO_3$ stock solution was added (the final concentration of Ag^+ is about 20 μM). Then, after about another 3min-incubation process under stirring, 8 μL freshly prepared $NaBH_4$ solution (1.0 mM) was added into the above solution (the final concentration of $NaBH_4$ is about 20 μM). Finally, after about another 6min-incubation process under stirring, 4 μL freshly prepared GSH solution (0.1 M) was added into the above solution (the final concentration of GSH is about 1.0 mM). Fluorescence emission was recorded at 570 nm with an excitation wavelength of 450 nm. (B) Gel images for demonstration of the reaction process shown in Scheme 1. The AgNPs/DNA-TPdye conjugate was prepared according to the experimental part. Lane 1: 200 nM DNA-TPdye; Lane 2: AgNPs/DNA-TPdye conjugate; Lane 3: AgNPs/DNA-TPdye conjugate with addition of GSH (the final concentration is 1.0 mM), the reaction mixture was incubated for 1 h at room temperature. Finally, all the mixtures were centrifuged at 12000 rpm and 15 μL of the obtained supernatants was used for gel running. Electrophoresis condition: 15% polyacrylamide gel and the gel was run at 200 V for 1 hrs, gel image was carried

out with a ChemiDoc XRS$^+$ Imaging System (Bio-RAD).

Fig. 4 (A) The fluorescence emission spectra of AgNPs/DNA-TPdye conjugate in responding to different concentration of GSH. The arrows indicate the signal changes as increases in GSH concentrations (From bottom to up: 0, 0.62, 1.25, 1.87, 2.50, 3.12, 3.75, 5.00, 5.62, 6.25, 7.50, 8.75, 10.00, 11.25, 13.75, 16.25 and 18.75 μM). Inset: GSH concentration-dependent change in F_0/F. Where F and F_0 are the fluorescence intensities of AgNPs/DNA-TPdye conjugate with and without adding GSH, respectively. (B) F/F_0 of the sensing system in responding to 12.50 μM of different amino acids, Cys, and Hcy. Where F and F_0 are the fluorescence intensities of AgNPs/DNA-TPdye conjugate with and without adding various biological molecules, respectively. For all the experiments, 400 μL of the diluted AgNPs/DNA−TPdye conjugate prepared as described in the experimental section was used and the experimental process was according to the experimental part. λ_{ex} = 450 nm.

Fig. 5 TP confocal fluorescence microscopy images of HeLa cells after incubating with (a) DNA-TPdye, (b) AgNPs/DNA-TPdye conjugate, and for (c) the cell was first treated by NMM (1.0 mM) before incubating with AgNPs/DNA-TPdye conjugate. (1) bright-field images, (2) Fluorescence images, (3) overlap of fluorescence and bright-field images. The used AgNPs/DNA-TPdye conjugate was prepared according to the experimental part.



Scheme 1

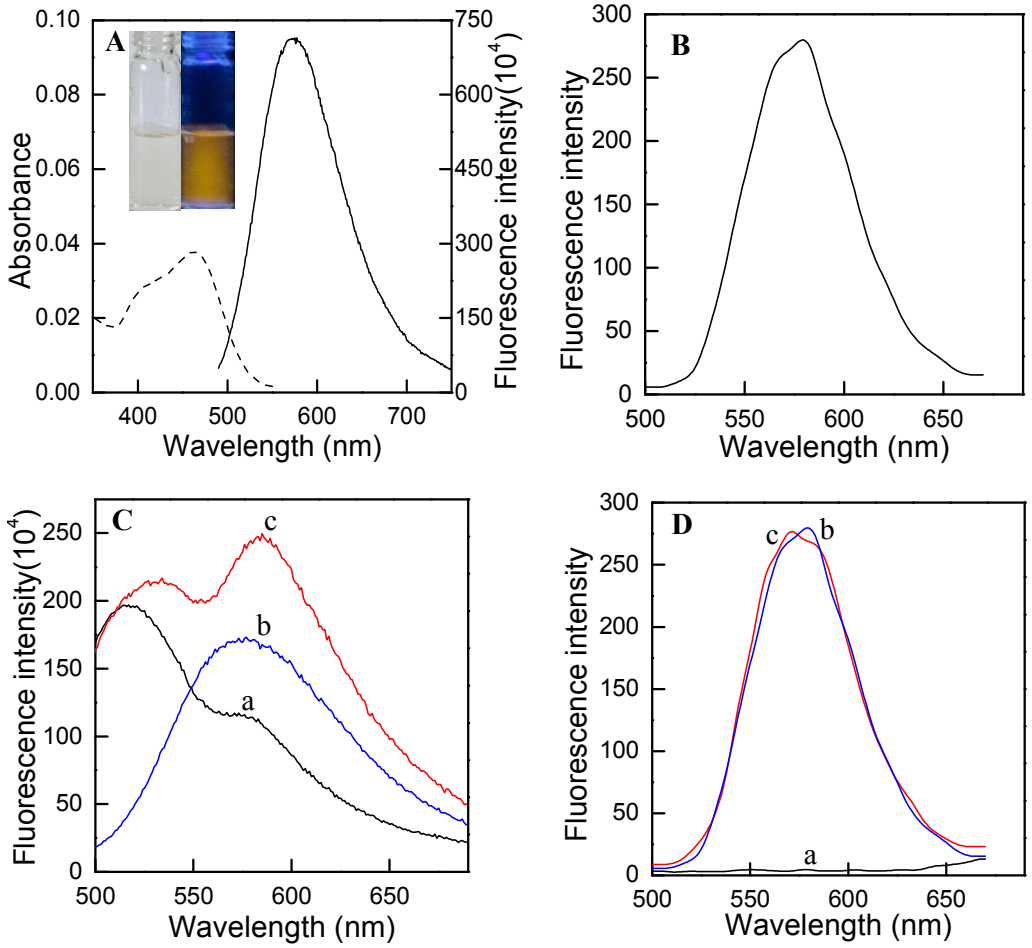


Fig. 1

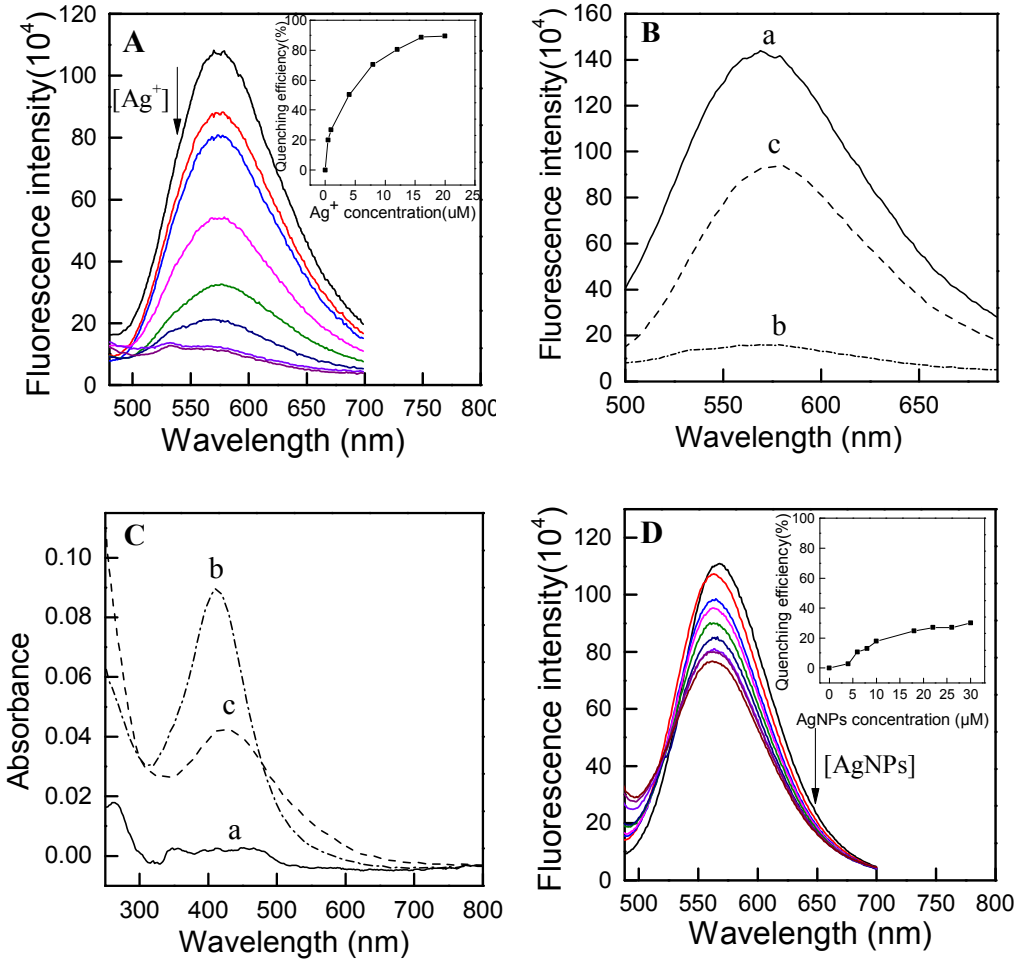


Fig. 2

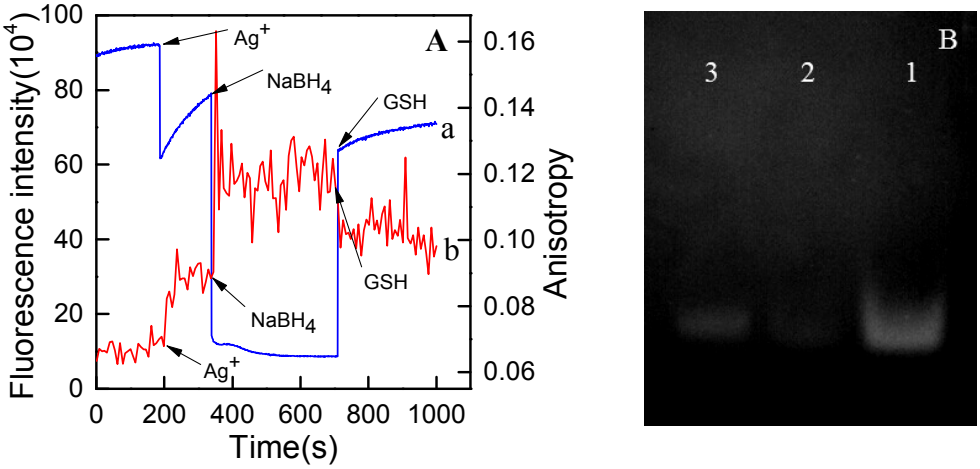


Fig. 3

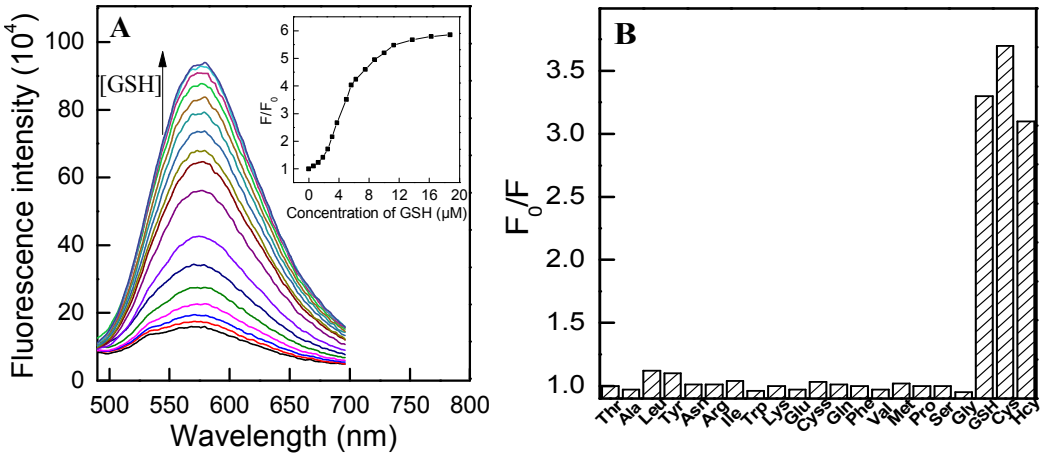


Fig. 4

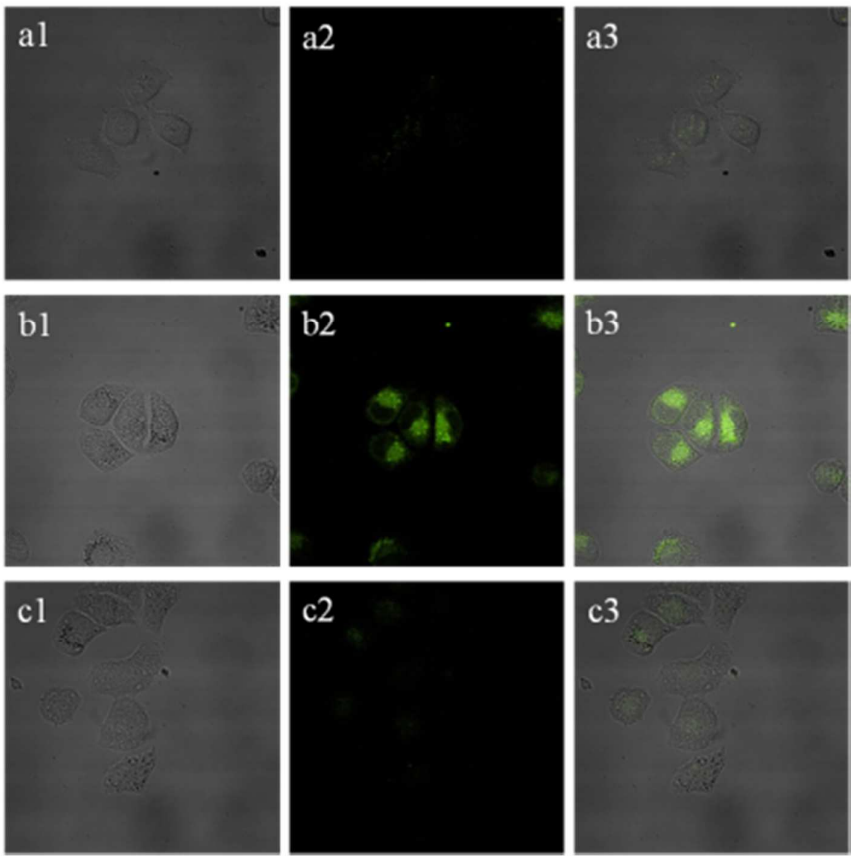
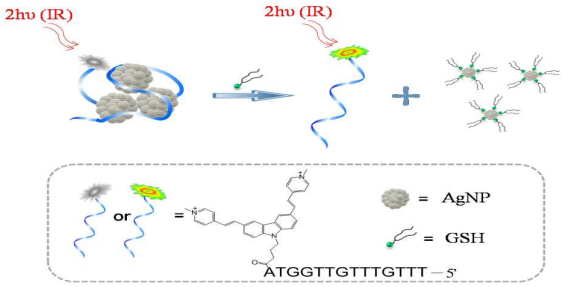


Fig.5

TOC



In this paper, we fabricate a novel AgNPs/DNA−two-photon dye (TPdye) conjugate as two-photon nanoprobe for biothiols imaging in live cells.

Electronic Supplementary Information

Two-photon AgNPs/DNA-TPdye Nanosensing Conjugate for Biothiols Probing in Live Cells

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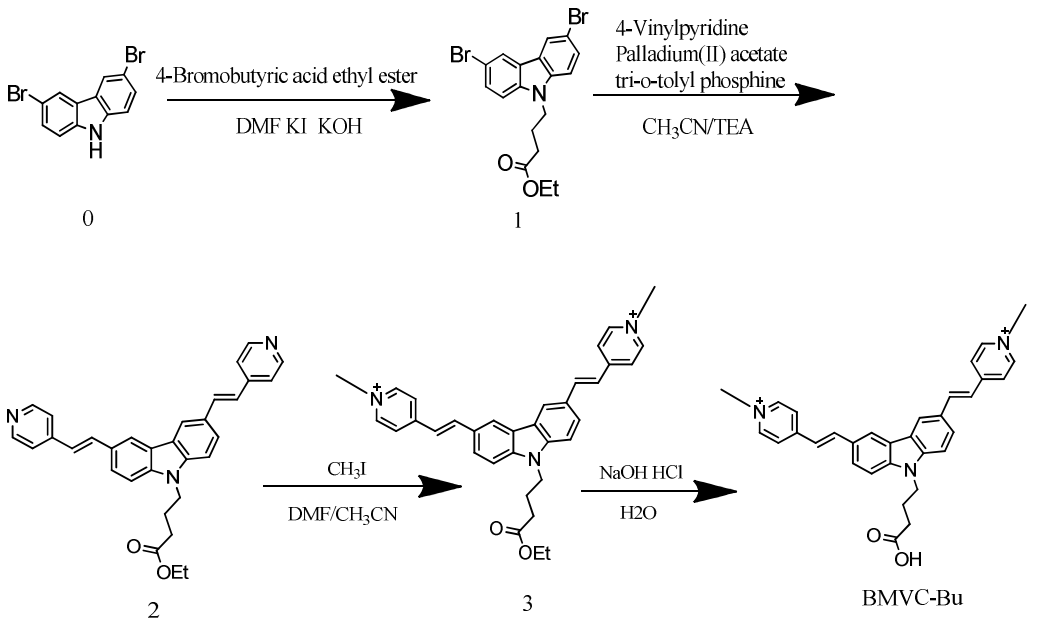
Preparation of the Two-photon Dye (TPdye: 4-[3,6-Bis(1-methyl-4 -vinylpyridium iodine)-9H-carbazol-9-yl)] butanoic acid, BMVC-Bu).



To a mixture of KOH (1.12 g, 20 mmol) and KI (80 mg, 0.48 mmol) dissolved in dry DMF (20mL) was added 3,6-dibromocarbazole (compound 0) (0.65g,2mmol), ethyl-4-bromobutanoate (1.15 mL, 8 mmol). The mixture was stirred at 60 ^{0}C under argon atmosphere overnight. After the addition of 100 mL H_2O to the final mixture, the mixture was extracted with ethyl acetate and then the organic layer was washed twice with water and once with brine, and dried over anhydrous Na_2SO4. After filtration, the solution was concentrated under reduced pressure to give crude product The final white powder Ethyl-4-(3,6-dibromo-9H-carbazol-9-yl) butanoate (compound 1) was obtained by chromatography using petroleum/ethyl acetate(5:1, V/V) as an eluent.[1]

4-[3,6-Bis(1-methyl-4-vinylpyridiumiodine)-9H-carbazol-9-yl)] butanoic acid was prepared as indicated in literature.[2] Ethyl-4-(3,6-dibromo-9H-carbazol-9-yl) butanoate (compound 1) (731.7 mg,1.7 mmol) was added into a mixture containing 4-vinylpyridine (667 mg), Palladium(II) acetate (5 mg) and tri-o-tolyl phosphine (50 mg) under the triethylamine (3 mL)/acetonitrile(9 mL) solvent pairs in a high pressure

bottle. The mixture was stayed at 105 ^{0}C for 48 h. After the reaction, the mixture was transferred to a flask and the solvent was removed under reduced pressure to give a yellow crude product, which was purified by chromatography on silica gel using CH_2Cl_2/CH_3OH (5:1,V/V) as an eluent to give Ethyl-4-[3,6-Bis(4-vinylpyridium iodine)-9H-carbazol-9-yl)] butanoate (compound 2) as earth yellow solid. Excess CH_3I and Ethyl-4-[3,6-Bis(4-vinylpyridium iodine)-9H-carbazol-9-yl)] butanoate (487.0 mg,1 mmol) in acetonitrile /DMF was refluxed for 4h,the orange red powder. Ethyl-4-[3,6-Bis(1-methyl-4-vinylpyridium iodine)-9H-carbazol-9-yl)] butanoate (compound 3) was obtained with a 90% yield after recrystallization twice using methanol. Then, Ethyl-4-[3,6-Bis(1-methyl-4-vinylpyridium iodine)-9H-carbazol -9-yl)] butanoate (compound 3) (193.0 mg, 0.25 mmol), sodium hydroxide (0.030 g, 0.75 mmol) were put into a 100mL flask containing the mixture solution of THF (4 mL) and water (1 mL) and the mixture was refluxed for 12 h. The diluted hydrochloric acid was added into the mixture to adjust pH=3 giving an orange red solid with a yield of 80%. ^{1}HNMR (d6-DMSO, 400 MHz, δ): 12.5 (s, 1H), 8.84 (d, 4H), 8.62 (s, 2H), 8.24 (d, 4H), 8.22 (d, 2H), 7.96 (d, 2H), 7,80 (d, 2H), 7.60 (d, 2H), 4.35 (t, 2H), 4.25 (s, 3H), 4.24 (s, 3H), 2.38 (t, 2H), 2.10 (m, 2H). MS (ESI) *m/z* for $C_{32}H_{31}N_3O_2$ 244.5 found, 244.5 (M^{2+}). Anal. calcd. for $C_{32}H_{31}I_2N_3O_2$: C, 52.19; H, 4.20; N, 5.65. Found: C, 52.19; H, 4.20; N, 5.64.

References

[1] X. B. Yan, S. P. Cheng and X. G. Chen, *Polymer.*, 2012, 53, 241-247.

[2] X. J. Feng, P. L. Wu and M. S. Wong, *Org. Lett.*, 2010, 12, 2194-2197.

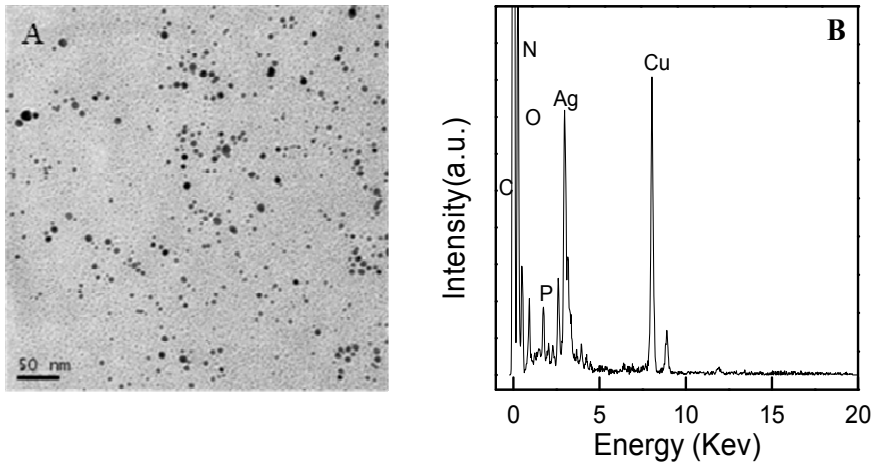


Figure S1. TEM image (A) and EDX spectroscopy analysis (B) of the AgNPs/DNA-TPdye conjugate.

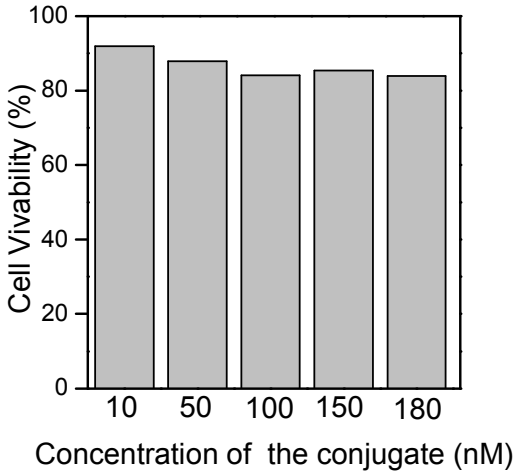


Figure S2. Cell viability of HeLa treated with different concentrations of AgNPs/DNA-TPdye conjugate for 24 h in fresh medium. The AgNPs/DNA-TPdye conjugate mixtures with different concentrations were prepared by mixing 946 μL of the fresh cell growth medium with 54 μL of 0.2, 0.9, 1.9, 2.8 and 3.3 μM AgNPs/DNA−TPdye conjugate prepared in the experimental section, respectively. All error bars were obtained through the detection of eight parallel samples. Note: The concentration of the conjugate refers to the concentration of DNA-TPdye.

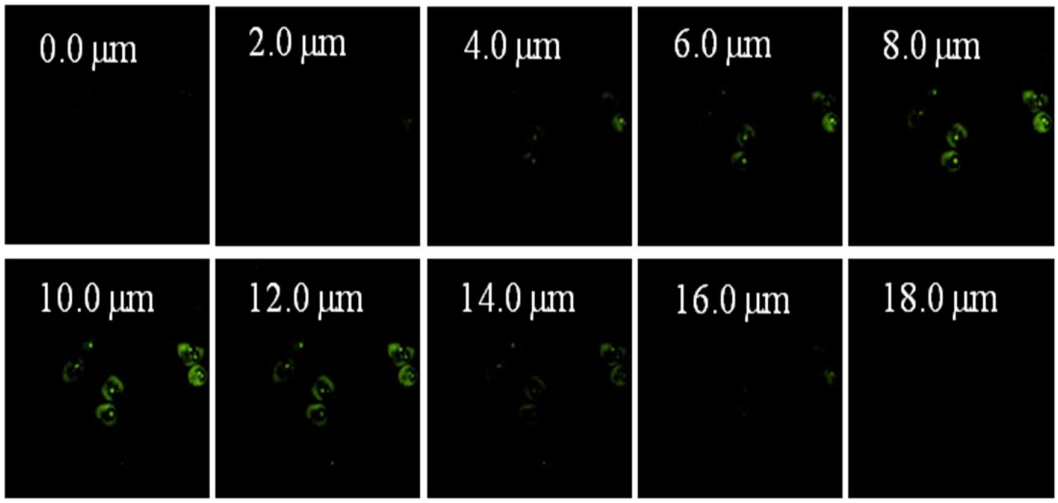


Figure S3. Z-scanning confocal fluorescence microscopy images of HeLa cells incubated with AgNPs/DNA-TPdye conjugate.