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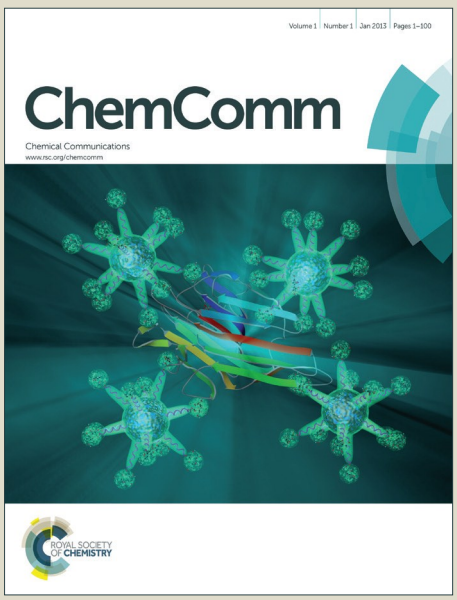





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Cite this: DOI: 10.1039/c0xx00000x

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ARTICLE TYPE

# AgNPs/DNA/TPdye Conjugate-based Two-photon Nanoprobe for GSH Imaging in Cell Apoptosis of Cancer Tissue

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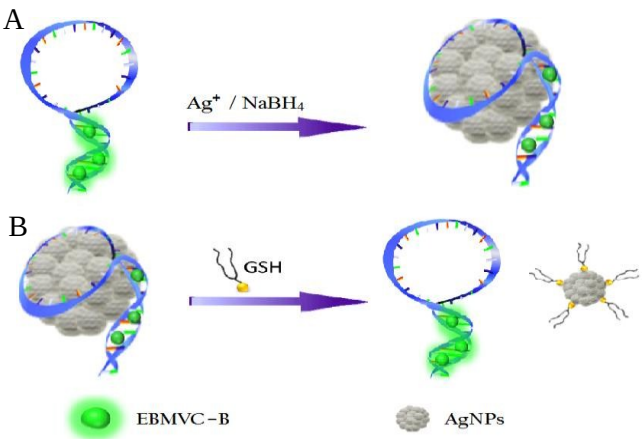
***Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX***
**DOI: 10.1039/b000000x**

**In the present study, a novel two-photon nanoprobe has been developed and successfully applied in Glutathione (GSH) imaging in cell apoptosis of cancer tissue.**

Glutathione (GSH), the most abundant thiol species in the cytoplasm and the major reducing agent in biochemical processes, serves as a very important mediator in many celluar functions.[1] Especially, as one of the most important anti-ROS (reactive oxygen species) systems, its abnormal level has a close connection with cell proliferation, cell apoptosis and cancers.[2] Due to its important roles in physiological activities, great attentions have been paid to detection of GSH in living cells and animals.[3] Among the present assays for GSH, fluorescence-based detection has received much attention for its high sensitivity and great simplicity in biological imaging. However, most of the reported fluorescent methods are based on one-photon excited (OPE) fluorophores,[4] which inevitably produce much larger self-absorption, autofluorescence, photodamages, and low tissue penetration depth 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 bioimaging applications.[7] Therefore, the TPE-based fluorescent sensing strategies will have a fascinating prospect for the in vitro or in vivo assay of GSH in complicated biological conditions.

Herein, considering the excellent properties of the TPE-based technique, and as a continuation of our studies on oligonucleotide/nanostructure conjugate-based sensing designs,[8] we constructed a novel AgNPs/DNA/Two-photon dye (TPdye) nanoprobe for GSH imaging in cell apoptosis of cancer tissues (Scheme 1). The nanoprobe is composed of a judiciously designed hairpin DNA (HPDNA, 5'-GGT TAA TCC AAG AAT CAA TAA CTA CAT AAG GAT TAA CC-3'), the duplex portion of which is used to load the TPdyes (EBMVC-B, see ESI),[9] and its single-stranded loop portion serves as the scaffold for AgNPs growth in aqueous solution.[10] The nanoprobe initially emits negligible fluorescence due to the efficient quenching of AgNPs to the TPdyes adjacent to the noble metal surface. GSH can replace the TPdye-embedded hairpin-DNA off the AgNPs surface via the strong thiol-Ag interaction, turning on fluorescence of the TPdyes.




**Scheme 1**. Schematic illustration of the sensing mechanism of the two-photon AgNPs/DNA/TPdye nanoprobe. Formation (A) and sensing mechanism (B) of the nanoprobe.

To verify this design scheme, we investigated the real-time records of OPE fluorescence intensity and the anisotropy changes of EBMVC-B/HPDNA complex in the HEPES buffer solution upon formation of AgNPs and subsequently addition of GSH (Figure 1A). In aqueous solution, EBMVC-B/HPDNA has a strong fluorescence emission, while the fluorescence emission was quenched greatly after the formation of AgNPs (curve a), indicating the interaction of TPdye EBMVC-B 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 EBMVC-B/HPDNA under the same conditions. The FA value of EBMVC-B/HPDNA complex at the free state in the buffer is low (0.17). However, it underwent an enhancement (0.38) upon formation of AgNPs. This result indicates that the DNA-templated synthesis of AgNPs created a larger mass conjugate, which hindered the rotation diffusion rate of the TPdye EBMVC-B.[11] The significant difference in the FA values of TPdye/DNA and the AgNPs/DNA/TPdye further confirms the formation of

AgNPs/DNA/TPdye nanoprobe. The FA value was reduced from 0.38 to 0.20 with addition of GSH, meaning that the strong competitive assembly of GSH on the AgNPs surface freed the TPdye/DNA complex from the AgNPs surface. It is worth noting that the fluorescence intensity and anisotropy changes of AgNPs/DNA/TPdye nanoprobe 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 nanoprobe was also performed by using gel electrophoresis (Figure 1B). The results show that there is no significant EBMVC-B/HPDNA band on the gel image for the supernatant of AgNPs/DNA/TPdye conjugates, while for the supernatant of AgNPs/DNA/TPdye conjugates with addition of GSH, there is a clear EBMVC-B/HPDNA band on the gel image, indicating that the TPdye/DNA 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.

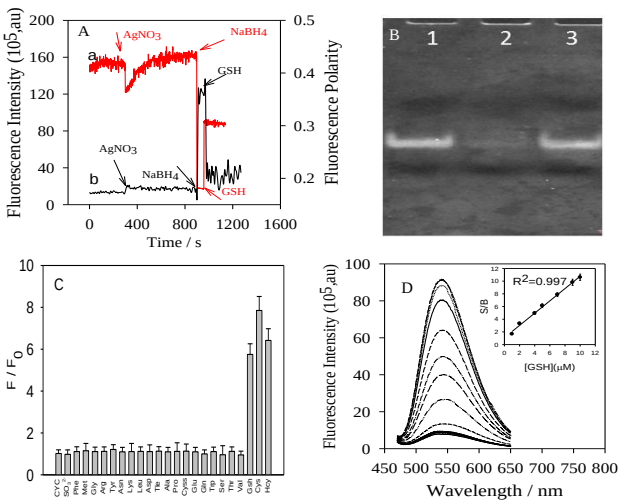



**Fig. 1** (A) Real-time fluorescence records (curve a) and fluorescence polarization changes (curve b) of EBMVC-B/HPDNA complex upon the formation of AgNPs/DNA/TPdye conjugate and addition of GSH. The transitions between each regime are marked with an arrow. (B) Gel image for demonstration of the reaction process shown in Scheme 1. Lane 1: 500 nM/100 nM of EBMVC-B/HPDNA; Lane 2: AgNPs/DNA/TPdye nanoprobe; Lane 3: AgNPs/DNA/TPdye nanoprobe with addition of GSH (the final concentration is 1.0 mM). (C) $F/F_0$ of the sensing system in responding to 100 μM of different amino acids. Where F and $F_0$ are the fluorescence intensities of AgNPs/DNA/TPdye nanoprobe with and without adding various biological molecules, respectively. (D) Fluorescence emission spectra of AgNPs/DNA/TPdye nanoprobe in responding to different concentration of GSH. Inset: GSH concentration-dependent change in S/B. Where S and B are the fluorescence intensities of AgNPs/DNA/TPdye nanoprobe with and without adding GSH, respectively. Fluorescence emission was recorded at 550 nm with an excitation wavelength of 450 nm.

To evaluate the selectivity of the prepared AgNPs/DNA/TPdye TP nanoprobe, the TPdye EBMVC-B fluorescence intensity changes with the addition of different targets (GSH and other amino acids) were studied. As shown in Figure 1C, when GSH and Cys/Hcy were presented, the nanoprobe system gives a significant fluorescence emission enhancement, while clear increase in fluorescence emission cannot 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. We then further investigated the capabilities of the AgNPs/DNA/TPdye nanoprobe as a high-performance fluorescent sensor for in vitro assay of GSH. The results show that a linear response in the range of 1~10 μM GSH with a detection limit of 0.3 μM (3σ rule) can be readily achieved (Figure 1D), and the TPE method is more suitable for biological assays compared with the OPE-based methods, owing to the low background fluorescence (Figure S1, ESI).

Before application of the DNA/AgNPs/TPdye nanoprobe for GSH imaging in cell apoptosis, the feasibility of the sensing nanoprobe for GSH detection was first demonstrated by using human cervix carcinoma (HeLa) cells as the model. As shown in Figure 2, HeLa cells incubated with the DNA/AgNPs/TPdye nanoprobe for 30 minutes at 37 $^{o}$C gave out strong fluorescence emission in the TPE fluorescence images, indicating that the DNA/AgNPs/TPdye nanoprobe successfully entered the cells and a relative high level of GSH in HeLa cervical carcinoma cells. In contrast to this, if the cells were first treated by using N-methylmaleimide (NMM, a GSH scavenger)[12] before incubating with the nanoprobe, no appreciable contrast in the fluorescence images can be observed. To further verify the internalization of DNA/AgNPs/TPdye nanoprobe in HeLa cells, Z-scanning confocal imaging was performed (Figure S2, ESI). It is clear that bright fluorescence was present throughout the whole cells, which suggested efficient delivery of the DNA/AgNPs/TPdye nanoprobe in the cytosol. Taken together, these revealed that the prepared DNA/AgNPs/TPdye nanoprobe can be successfully used for GSH imaging in live cells.

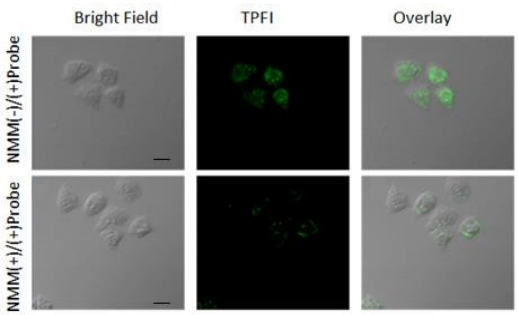



**Fig. 2** TPM images of HeLa cells treated with(c)/without(b) NMM.

After interrogating the response characteristics of the DNA/AgNPs/TPdye nanoprobe for GSH detection in cells, we monitored GSH variation in a 1.0 mm-thick living cervical tumor tissue slice pretreated with or without PEITC, an element extracted from some plants which can induce cell apoptosis through decreasing the level of reducing agents and thus destroying the intracellular redox equilibrium,[13] by using TP confocal microscope for fluorescence emission detection after the nanoprobe incubation. The 3D TP fluorescence images showed that the DNA/AgNPs/TPdye nanoprobe provided high TPE fluorescence emission signal for the tumor tissue slice without PEITC treatment (Figure 3B), while the TPE fluorescence emission signal from the PEITC-treated tumor tissue slice was substantially weaker (Figure 3A), indicating the significant

decrease of GSH level and the enabled clear visualization of apoptosis in cervical tumor tissue. Overall, our data presented show that the prepared DNA/AgNPs/TPdye nanoprobe can be used for high-contrast TPE GSH imaging in apoptotic and deep tumor tissues.

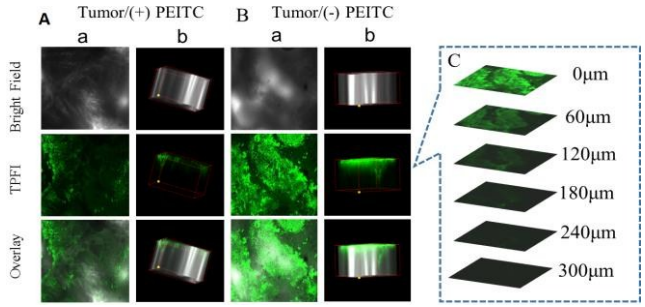



**Fig. 3** TPM images of the 1.0 mm-thick cervical tumor tissue slice pretreated with (A) or without (B) PEITC after incubating with AgNPs/DNA/TPdye nanoprobe; (a) The TPE images at a depth of 0 μm and (b) the corresponding 3D images accumulated along the Z-direction at depth of 0–300 μm. (C) The confocal Z-scan TPFI sections of the nanoprobe-incubated tumor tissue slice at different penetration depths.

In conclusion, we have developed a TPE-based sensor for detecting GSH in live cells and tissues. The sensor is prepared via formation of the DNA-templated AgNPs and binding a TPdye to the DNA. The TPdye/DNA/AgNPs conjugate exhibits desirable two-photon-sensitized fluorescence properties, good cell-permeability, high stability and good biocompatibility. Compared with the nowadays prevailing strategy of reaction or DNA molecular beacon-based OPE biosensor construction, the introduction of a two photon dye as the signal reporter effectively eliminates problems of self-absorption and autofluorescence in biological matrixes while using long excitation wavelength, and offers high, larger depth penetration, as well as less photodamage. Moreover, the conjugation of DNA/TPdye with silver nanoparticles by DNA-templated synthesis is simple without the need of either surface functionalization of the nanoparticles or covalently labeling the DNA. In vitro and in vivo assays revealed that the DNA/AgNPs/TPdye nanoprobe provided a robust, sensitive, and selective sensor for quantitative detection of GSH even under complex biological conditions. To the best of our knowledge, it is the first time that a DNA/AgNPs/TPdye two-photon nanoprobe has been successfully used for biothiols assay in live cells and tissues. Considering the physiological link between GSH and a variety of diseases and disease status, this new DNA/AgNPs/TPdye TPA nanoprobe is expected to hold great potential for in vitro and in vivo applications in anti-cancer drug screening, medical research and clinical diagnostics.

The authors would like to acknowledge the financial support from NSFC (21475036, 21205143, 21205039, 21135001, and J1103312) and the ‘‘973’’National Key Basic Research Program (2011CB91100-0). The authors also thank Professor Zhihong Liu of Wuhan University for the TPE spectra measurements.

## Notes and references

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*†Electronic Supplementary Information (ESI) available: Experimental details, characterization of the synthesized TPdye and TPdye/DNA complex, formation and cytotoxicity of the AgNPs/DNA/TPdye nanoprobe and other additional information as noted in text. See DOI: 10.1039/c000000x/*

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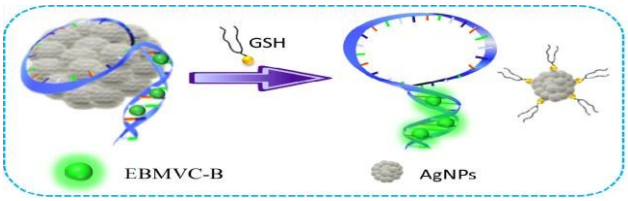


By combination of DNA with two-photon dye (TPdye) and silver nanoparticles (AgNPs), a novel two-photon nanoprobe for Glutathione (GSH) imaging in cell apoptosis of cancer tissue was designed in the present study.