Supplementary Information

A Highly Selective and Sensitive Nanoprobe for Detection and Imaging of Superoxide Anion Radical in Living Cells

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Materials: All reagents were of commercial quality and used without further purification. 3-aminopropyl-trimethoxysilane (APTS), O-aminobenzenothiol (99%), *S*-Nitroso-*N*-acetyl- DL-penicillamine (SNAP), Hepes, phorbol 12-myristate 13 acetate (PMA), Glutathione (GSH), Sodium Nitroferricyanide(III) Dihydrate (SNP), Xanthine Oxidase (XO), Xanthine (XA), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), 3-(aminopropyl)-1-hydroxy -3-isopropyl-2-oxo-1-triazene (99%) were purchased from Sigma Chemical Company. 4, 5-dihydroxy-1, 3-benzenedisulfonic acid disodium salt (Tiron) was from Shanghai Reagent Co. Ltd. $O_2^{\bullet-}$ was initiated through the enzymatic reaction of XA/XO, OH• and single oxygen ($^1O_2$) was generated by reacting H_2O_2 with Co^{2+} or NaClO. Nitric oxide (NO) and peroxynitrite ($ONOO^-$) were obtained from 3- morpholinosydnonimine hydrochoride (SNP-1) and 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene, respectively. 2-chloro-1,3-dibenzothiazolinecyclohexene (DBZTC, synthesized in-house) was prepared in our lab.[1] Sartorius ultrapure water (18.2 MΩ cm) was used throughout the analytical experiments. RAW 264.7 macrophage cells were purchased from the Committee on Type Culture Collection of the Chinese Academy of Sciences. ^1H

NMR, FTIR and Elemental Analysis were employed to characterize DBZTC. The data are consistent with the previous report [1] and are shown as following: ^{1}H NMR (300 MHz, DMSO-d_6, 25ºC, TMS): δ 8.10-7.90 (m, 2H, benzol-H), 7.75 (d, 2H, benzol-H), 7.47-7.39 (m, 2H, benzol-H), 7,24 (d, 2H, benzol-H), 4.10 (m, 2H, N-H), 3.12 (m, 2H, methine-H), 2.3 (m, 3H, cyclohexene-H), 1.15 (m, 4H, cyclohexene-H). IR (KBr pellet): ν (cm^{-1}) 3304 (N-H), 3053 (C-H), 1582 (C=C). Melting point: 95~97ºC. Elemental analysis (%) calcd for $C_{20}H_{19}N_2S_2Cl$ (found): C 62.10 (62.02), H 4.92 (4.84), N 7.24 (7.36).

Apparatus and Measurements: Transmission electron microscopy (TEM) was carried out on a JEM-100CX Π electron microscope. Absorption spectra were measured on a pharmaspec UV-1700 UV-visible spectrophotometer (SHIMADZU). Fluorescence spectra were obtained with FLS-920 Edinburgh Fluorescence Spectrometer with a Xenon lamp and 1.0 cm quartz cells at the slits of 2.5/2.5 nm. All pH measurements were performed with a pH-3c digital pH-meter (Shanghai LeiCi Device Works, Shanghai, China) with a combined glass-calomel electrode. ^{1}H spectra were taken on a Bruker 300-MHz spectrometer. Determination of organic elements was obtained with Model PE-2400(II) element analyzer. Absorbance was measured in a TRITURUS microplate reader in the MTT assay. The fluorescence images of nanocomposites and cells were taken using a LTE confocal laser scanning microscope (Germany Leica Co., Ltd). Fourier transform infrared (FTIR) spectrum was performed using a Bruker Tensor-27 spectroscope with KBr pellets. All pH measurements were performed with a pH-3c digital pH meter (Shanghai Lei Ci Device Works, Shanghai, China) with a combined glass–calomel electrode.

Preparation of Ag NPs, Ag@SiO_2 and DBZTC/Ag@SiO_2: $AgNO_3$ (9 mg) was dissolved in 100 ml H_2O and brought to boiling, and 2 ml of 1% sodium citrate solution was added. The solution was kept on boiling for ca. 1 h. Then Ag NPs were purified by centrifugation at 500 rpm for 1 h to remove the larger NPs. Next, 10 ml of Ag NPs solution was added to 20 ml ethanol and added ammonium hydroxide to pH=10 with strong stirring for 5 minutes. Then different concentrations of TEOS (0.5 mM to 3 mM) were added to initiate SiO_2 layer (TEOS was dispersed in 15 ml

ethanol) every 30 minutes with strong stirring once for 1 ml, and reacted for 24 h. Without further purification, 3-aminopropyltriethoxysilane (APTS, a silane coupling agent) was added to the solution with 24 hours of stirring to initiate $–NH_2$, in which the amount of APTS was the 1/10 of the volume of solution. The nanoparticles were collected by centrifugation and washed by water and ethanol for three times, and dispersed in water.

To certify the existence of $–NH_2$ groups, ninhydrine was employed. Ninhydrine can react with primary amine and results in a blueviolet or purple solution. When ninhydrine was added to the $Ag@SiO_2$ NPs solution, the color remained yellow (Fig. S2a). After the treatment with ninhydrine, the functionalized $Ag@SiO_2$ NPs solution gave a purple color (Fig. S2b). In this experiment, 1% of ninhydrine was added to 3 ml $Ag@SiO_2$ solution and brought to boiling. In order to confirm $–NH_2$ groups were modified onto the surface of the $Ag@SiO_2$ NPs instead of in the solution, another experiment was performed in the supernatant liquid of the centrifuged functionalized $Ag@SiO_2$ NPs solution. As can be seen in Fig. S2c, the solution is colorless. It suggests $Ag@SiO_2$ NPs are successfully functionalized with $–NH_2$ groups. Finally, DBZTC was loaded onto the $Ag@SiO_2$ surface through the reaction of $–NH_2$ and –Cl to detect superoxide anion radical. 1 ml of 10 mM DBZTC (excess) was added to 5 ml of $Ag@SiO_2$ solution, and incubated in 80 °C under Ar atmosphere for 24 h. After the reaction, there were DBZTC and DBZTC/$Ag@SiO_2$ nanocomposite in the solution. DBZTC/$Ag@SiO_2$ nanocomposite was collected by centrifugation. Then the nanocomposite was washed for three times with DMSO and water, and dispersed in 1 ml water. The concentration of unreacted DBZTC was monitored by the absorption of the supernatant liquid. The final DBZTC concentration of the nanocomposite could be calculated to be 3.2×10^{-4} M (from initial DBZTC concentration before reaction and unreacted DBZTC concentration). The comparison sample APTS-DBZTC was prepared as below: 1 ml of 10 mM DBZTC was added to 5 ml of 2mM APTS solution, and incubated in 80 °C under Ar atmosphere for 24 h. Fig. S7 showed the fluorescence intensity of DBZTC was constant after the reaction between $–NH_2$ and –Cl. In the presence $O_2^{\bullet-}$, fluorescence intensities of DBZTC and

APTS-DBZTC were almost same. It indicated the fluorescence enhancement was not due to the reaction between $–NH_2$ and –Cl.

When DBZTC was added to NH_2–functionalized $Ag@SiO_2$ solution, the pH value was measured to be 7.20. After incubated in 80 °C for 24 h, the pH value was measured to be 6.26. The decrease of the pH value indicated the formation of HCl after the reaction. It suggested the loading of DBZTC on the surface of $Ag@SiO_2$ through the reaction between terminal $–NH_2$ and –Cl. Fig. S3 shows the laser scanning confocal fluorescence microscopy (CLSM) image of DBZTC/$Ag@SiO_2$ nanocomposite which are incubated with superoxide anion radical and excited with 480 nm wavelength. Bright green fluorescent particles with 250±50 nm were clearly observed, which showed little aggregation. The result demonstrates that DBZTC is successfully assembled onto the surface of $Ag@SiO_2$ NPs. It was further confirmed by FTIR of the nanocomposite: N-H stretch vibration at 3358 cm^{-1}, C-H stretch vibration in benzene at 3053 cm^{-1}, saturation C-H stretch vibration at 2829 $cm^{-1}$and 2858 cm^{-1}, C=C at 1370-1610 cm^{-1}, Si-O at 1089 cm^{-1}(Fig. S4). The appearance of the characteristic peaks [1] (C-H stretch vibration in benzene and C=C at 1370-1610 cm^{-1}) for DBZTC in the spectrum indicates the successful loading of DBZTC on $Ag@SiO_2$.

Cell Culture: Primary cultured macrophages (RAW 264.7) were grown in a cell-culture flask in high-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/mL penicillin and 100 U/mL streptomycin. Cultures were maintained in a humidified incubator at 37 °C, in 5% CO_2-95% air.

Confocal Fluorescence Image Assay: One day before imaging, cells were passed and plated on 18-mm glass cover slips in culture dish. Then the cells were stimulated by PMA for 12h. Prior to imaging, the medium was removed. Cell imaging was carried out after washing cells with Hepes (pH 7.4, 20 mM) for three times. All background parameters (the laser intensity, exposure time, objective lens) were stationary when the different fluorescence images were captured. Excitations of nanoprobe-loaded cells at 480 nm were carried out with a HeNe laser, and emission was collected using a META detector between 490 nm and 600 nm, respectively.

MTT Assay: To investigate cytotoxicity of the nanoprobe, MTT assay were carried out when the probes existed. RAW 264.7 macrophage cells (1×106 cells/well) were dispersed within replicate 96-well microtiter plates to a total volume of 200 μL well-1. Plates were maintained at 37 °C in a 5% CO_2/95% air incubator for 12 h. The probe was added to each well after the original medium has been removed. Macrophages were incubated with 5 μM nanoprobe for 0, 0.5, 1, 2, 6, 12, 24 h. Then 100 μL MTT solution (0.5 mg mL-1 in PBS) was added to each well. After 4 h, the remaining MTT solution was removed, and 150 μL of DMSO was added to each well to dissolve the formazan crystals. The absorbance was measured at 490 nm in a TRITURUS microplate reader.

Reference:

1 J. J. Gao, K. H. Xu, B. Tang, L. L. Yin, G. W. Yang, L. G. An, *FEBS Journal.* 2007, **274**, 1725.

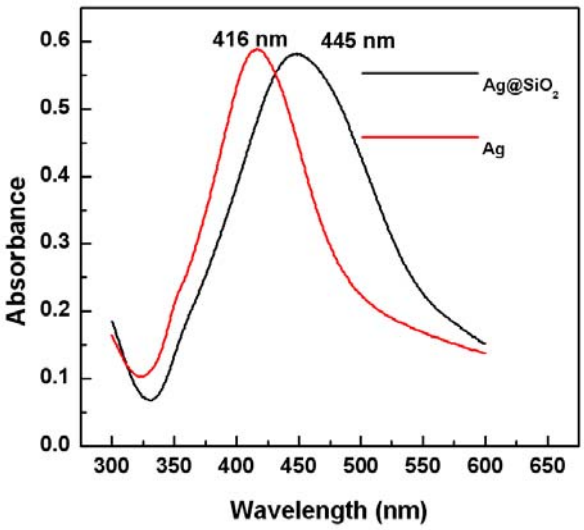

Fig. S1. UV-vis absorption spectra of Ag NPs and Ag@SiO_2 (the thickness of SiO_2 shell was 20 nm) in water.

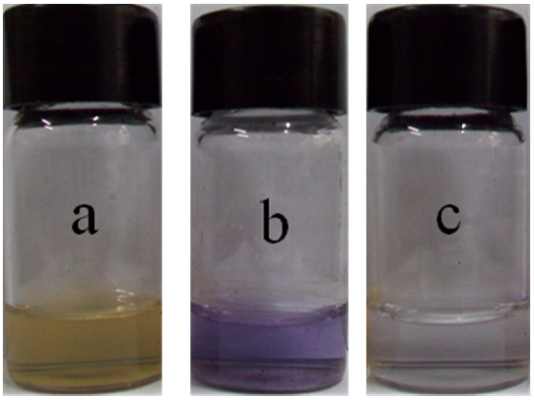

Fig. S2. Verification of amino-group (a. Ag@SiO_2 NPs without functionalization dispersed in water; b. Ag@SiO_2 NPs functionalized with –NH_2 dispersed in water; c. the supernatant liquid of b after centrifugation.).

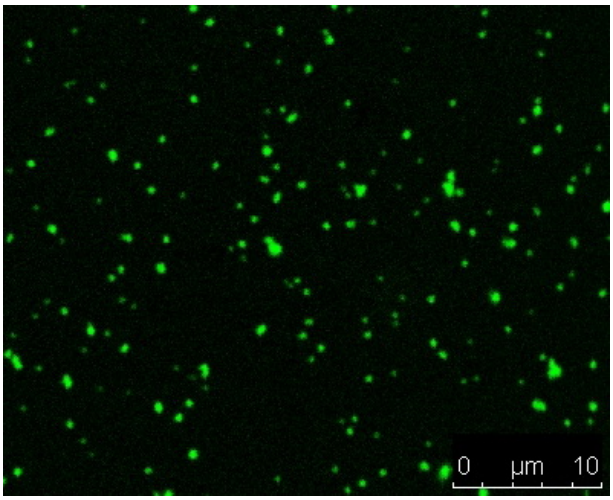

Fig. S3. Confocal image of DBZTC/Ag@SiO_2 nanocomposite (incubating with superoxide anion radical) in solution, obtained with 480 nm laser excitation. Scale bar is 10μm.

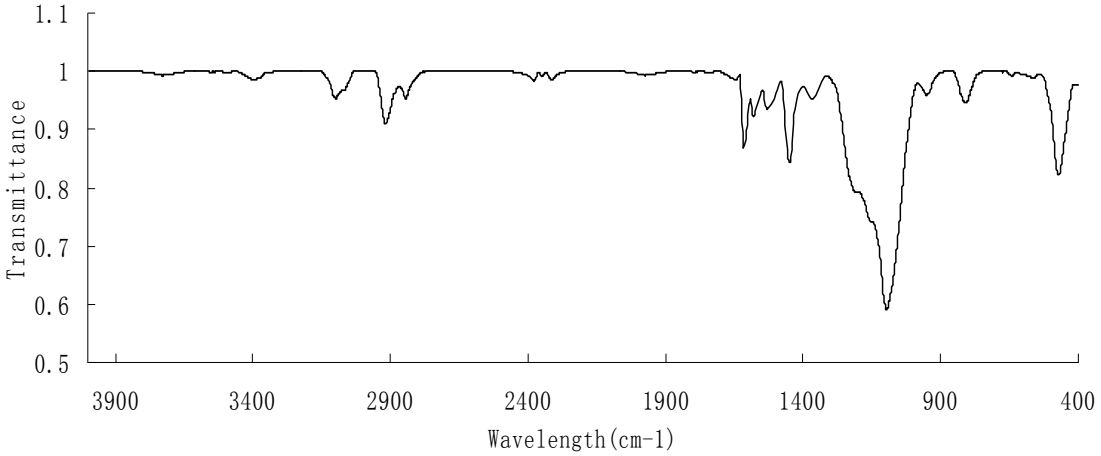

Fig. S4 FTIR spectrum of DBZTC/Ag@SiO_2 nanocomposite.

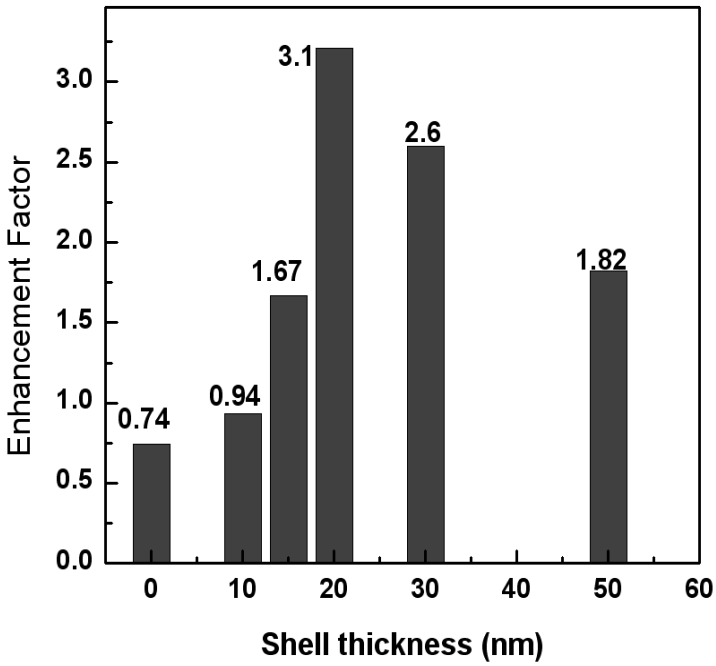

Fig. S5 Enhancement factor as a function of different SiO_2 shell thickness (0 nm, 10 nm, 15 nm, 20 nm, 30 nm, 50 nm). The fluorescent enhancement factor is defined as the ratio of the fluorescence intensity of DBZTC/Ag@SiO_2 nanocomposite to the fluorescence intensity of the same concentration of DBZTC toward 0.66 μM $O_2^{\bullet -}$. (Hepes buffer, pH= 7.4, 37 °C, 5 μM DBZTC . excitation=485 nm.)

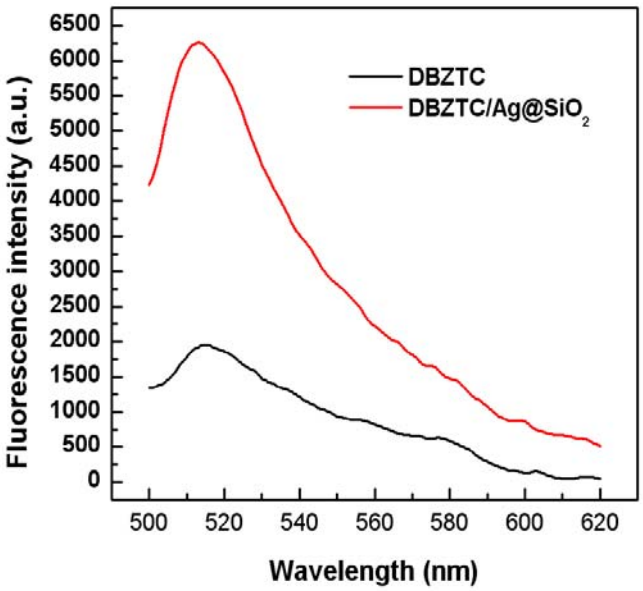

Fig. S6 Emission spectrum of DBZTC/Ag@SiO_2 nanocomposite (with 20 nm SiO_2 layer) toward 0.66 μM $O_2^{\bullet-}$, Ag@SiO_2 solution was chosen as blank; emission spectrum of DBZTC toward 0.66 μM $O_2^{\bullet-}$, buffer solution was chosen as blank. (Hepes buffer, pH= 7.4, 37 °C, 5 μM DBZTC, excitation=485 nm.)

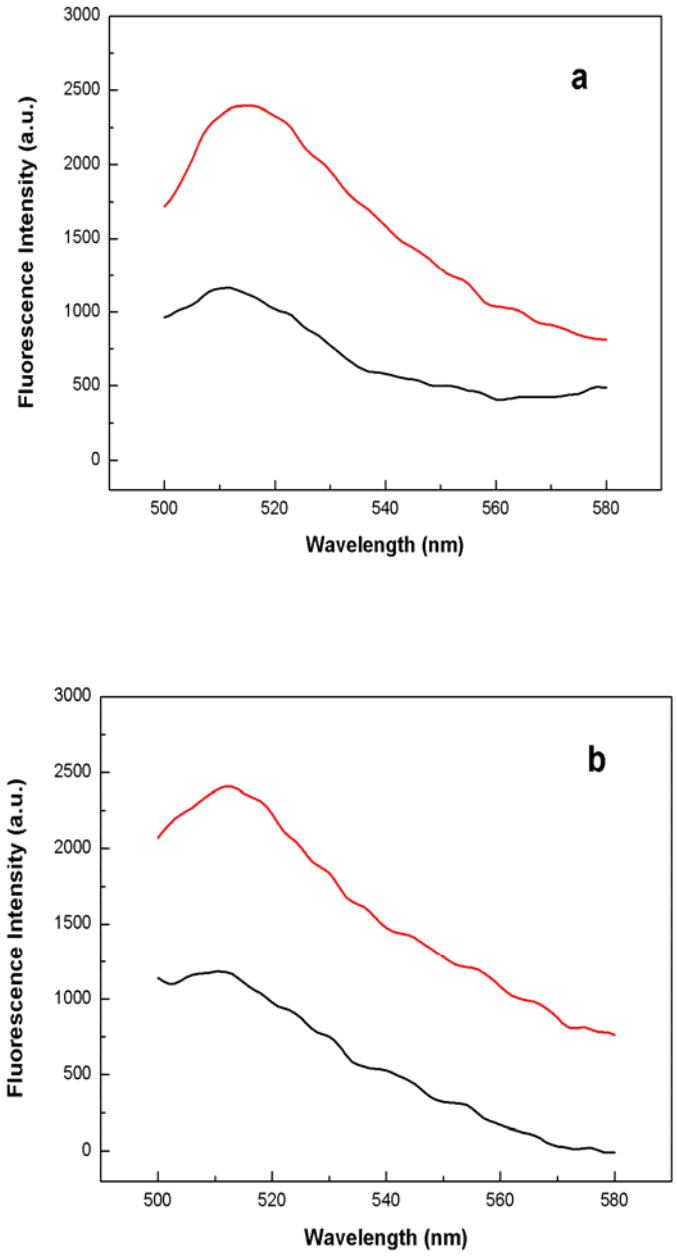

Fig. S7 a: Emission spectra of DBZTC (black line) and DBZTC toward 0.66 μM $O_2^{\bullet-}$ (red line), buffer solution was chosen as blank; b: Emission spectra of APTS-DBZTC (black line) and APTS-DBZTC toward 0.66 μM $O_2^{\bullet-}$ (red line), APTS buffer solution with same concentration was chosen as blank. (Hepes buffer, pH= 7.4, 37 °C, 5 μM DBZTC . excitation=485 nm.)

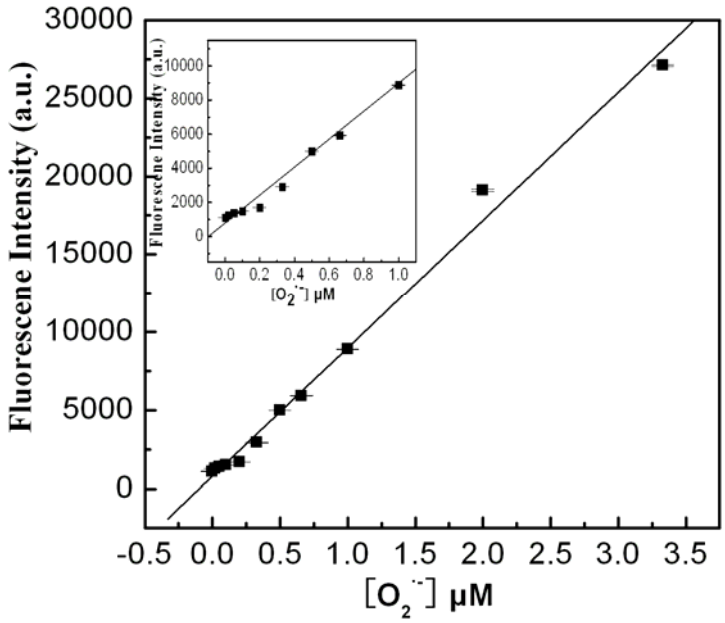

Fig. S8. Linear correlation between the fluorescence intensity and $O_2^{\bullet-}$ concentrations (0-3.33μM); Inset: linear correlation between the fluorescence intensity and $O_2^{\bullet-}$ concentrations (0-1μM). (The fluorescence intensity and $O_2^{\bullet-}$ concentrations were obtained from Fig. 3a).

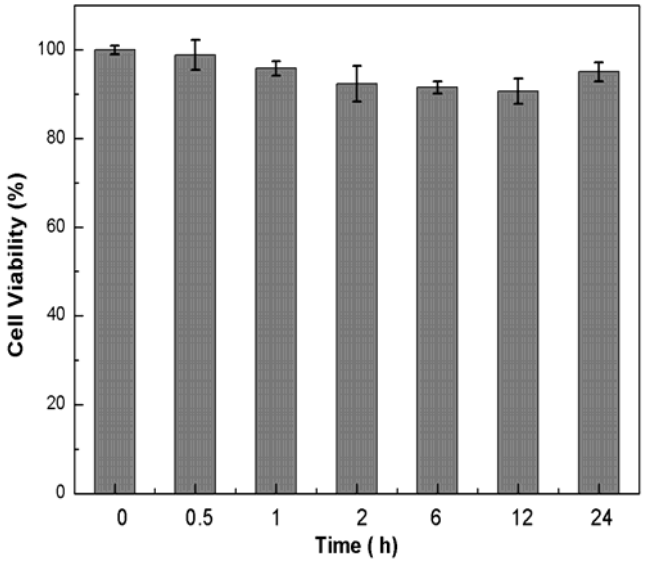

Fig. S9 Cell viability after incubation of RAW 264.7 cells with various time of DBZTC (5 μM) loading onto the nanocomposite. The percentage of cell viability calculated relatively to that of the cells without adding DBZTC/Ag@SiO_2 nanocomposite (shell thickness of 20 nm) is defined as a viability of 100%.