Supporting Information

In Situ Ratiometric Fluorescence Imaging for Tracking Targeted Delivery and Release of Anticancer Drug in Living Tumor Cells

Keqin Yang,†‡ Jingjin Zhao,† Liangliang Zhang,*,† Rongjun Liu,† Hong Liang,† Shulin Zhao*,†

†State Key Laboratory for the Chemistry and Molecular Engineering of Medicinal Resources, School of Chemistry and Pharmaceutical Sciences, Guangxi Normal University, Guilin, 541004, China
‡ Guilin Normal College, Guilin, 541001, China

Corresponding author: Professor Shulin Zhao and Liangliang Zhang
E-mail: zhaoshulin001@163.com
liangzhang319@163.com
EXPERIMENTAL SECTION

Preparation of BQDs. The BQDs were synthesized by hydrothermal heating of grapes in an autoclave. Briefly, 5 g of fresh grapes were placed in a mortar, ground, and transferred to a 50 mL Teflon-lined autoclave. Then 20 mL of ultrapure water was added into the Teflon-lined autoclave, and the autoclave was heated under stirring for 10 h at 220 °C. After the reaction mixture cooled to room temperature and ultrasonicated for 30 min, the supernatant was filtered with a 0.22 μm membrane, adjusted to pH≈7, and the obtained filtrate was then dialyzed for 24 h, through a dialysis bag (500 Da) in ultrapure water. The dialysate was stored at 4 °C for the following experiments.

Preparation of BQD-Aptamer (BQD-Apt) Nanoprobe. BQD solution (1000 μL) was added to 1225 μL of 10 mM borate buffer (pH=7.4) and mixed well by stirring. After 30 min, 200 μL of 100 μM 5′-amine terminated MUC1 DNA aptamer (5′-GCA GTT GAT CCT TTG GAT ACC CTG G-3′) were added to the reaction mixture under continued stirring. Then, 50 μL of 50 mM EDC and 25 μL of 25 mM NHS were added to the reaction mixture and incubated with gentle stirring for 4 h at room temperature protected from light. After the incubation, the mixture was centrifuged using Centrifugal Filter Unit with molecular weight cutoff of 10 kD for 10 min at 12,000 rpm, then washed three times using 50 mM borate buffer (pH=8.3) and reconstituted in 1 mL ultrapure H₂O for storage at 4 °C for future use.

DOX Loading and Releasing. In order to evaluate the capacity of the BQD-Apt nanocomposite probe for drug loading and release, we have selected DOX as a model anticancer drug. BQD-Apt were dispersed in DOX solution of a series of concentrations at room temperature. DOX molecules were loaded onto BQD-Apt stirring at 25 °C for 12 h, producing the BQD-Apt-DOX complex. The resultant BQD-Apt-DOX complex was then purified and washed with phosphate buffer solution (PBS) three times using 3 KD Nanosep centrifugal devices through centrifugation (12000 rpm, 10 min). The purified BQD-Apt-DOX complex was then stored at 4 °C for future use. The concentration of DOX loaded onto BQD-Apt was measured by the absorbance peak at 490 nm (calculated from the standard calibration curve). Drug loading content was calculated according to the following equation: Drug loading content=(W_{DOX in BQD-Apt})/(W_{BQD-Apt-DOX}), where W_{DOX in BQD-Apt} or W
BQD-Apt-DOX stands for the weight of DOX loaded onto BQD-Apt or the weight of the resultant BQD-Apt-DOX complex, respectively.

To measure DOX loading capacity, 200 μL DOX aqueous solution of concentrations from 30 to 210 μg/mL was mixed with 200 μL BQD-Apt (210 μg/mL) and stirred for 12 h. The prepared BQD-Apt-DOX complex was purified through centrifugation as mentioned above, producing the filter liquor containing residual free DOX. The resultant filter liquor was then used to determine the concentration of residual DOX based on the standard calibration curve obtained by UV-vis absorbance.

To investigate DOX in vitro pH-dependent releasing behavior, the prepared BQD-Apt-DOX complex (pH = 7.4) was dispersed in PBS solutions of different pH values (5.5, 6.8, and 7.4) for various times (1, 3, 6, 12, and 24 h) at 37 °C. DOX molecules detached from BQD-Apt surface were collected via centrifugation, which were then measured using UV-vis absorption spectrum to determine the amount of released DOX according to the standard calibration curve.

**Cells Incubation.** In the following experiments, HeLa cells, HepG2 cells, and 7702 cells were used as cell lines for high and low expression of mucin 1 protein on the surface of the cell membrane, respectively. Three cell lines were cultured at 37 °C in a humidified atmosphere with 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (100 units/mL streptomycin and 100 units/mL penicillin). All experiments were repeated three times in parallel.

**Ratiometric Fluorescence Imaging.** Cells (HeLa, HepG2 and 7702) were seeded in 35 mm confocal dish at 37 °C for 12 h under 5% CO₂ at 2.0 × 10⁵ cells per well. Then the cells were rinsed with fresh PBS (10 mM, pH=7.4) three times, following by co-incubation with the prepared BQD-Apt-DOX (prepared from 5 μg mL⁻¹ DOX) for different times (1, 3, 6, 9, 12, and 24 h) at 37 °C. After washing, the resultant sample was imaged using confocal laser scanning microscopy. For the ratiometric imaging, BQDs and DOX channels were both excited at 405 nm, and the emission windows of BQDs and DOX channels were 480-530 and 580-630 nm, respectively.

**Subcellular Localization of BQDs and DOX.** HeLa cells were seeded in 35 mm confocal dish at 37 °C for 12 h at 2.0×10⁵ cells per well, and then co-incubated with BQD-Apt-DOX (prepared from 5 μg mL⁻¹ DOX) for 12 h. After removing free and nonspecifically adsorbed BQD-Apt-DOX via washing with PBS three times, the cells were stained with Lyso-Tracker blue (100 nM) for 30 min. After washing, the
obtained sample was imaged using the confocal laser microscopy. Lyso-Tracker blue, BQD, and DOX channels were excited at 405 nm, 405 nm, and 488 nm, respectively. Emission windows of Lyso-Tracker blue, BQD, and DOX channels were set at 430-480 nm, 480-530 nm, and 580-630 nm, respectively.

For nuclear staining study, HeLa cells were co-incubated with BQD-Apt-DOX (prepared from 5 μg/mL DOX) for 12 h at 37 °C. The cells were then stained with Hoechst for 30 min and rinsed with PBS buffer before imaging. Hoechst, BQD, and DOX channels were excited at 405 nm, 405 nm, and 488 nm, respectively. The emission windows of Hoechst, BQD, and DOX channels were 430-480 nm, 480-530 nm, or 580-630 nm, respectively.

**In Vitro Tumor Cells-Targeted Therapy.** The cytotoxicity of the obtained BQDs and their compounds was assayed by MTT experiment. In brief, cells were cultured in 96-well plates at 1.5 × 10⁴ cells per well in DMEM for 24 h at 37 °C under 5% CO₂. Then, pure BQDs, free DOX, BQD-Apt, and BQD-Apt-DOX were mixed with cell medium with a serial concentration (from 5 to 200 μg/mL), and the mixture was added into the each well and co-incubated with cells for 24 h. After washing, 180 μL fresh culture medium and 20 μL MTT (5 μg/mL) were added. The culture medium was then removed after being incubated for 4h, and the obtained formazan crystals were dissolved in 100 μL DMSO and shaken for 10 min. The optical density (OD) of the mixture was measured using a Microplate Reader. The cell viability was estimated according to the following equation: Cell viability (%)=\(\frac{OD_{treated}}{OD_{control}} \times 100\%\), where \(OD_{control}\) was obtained in the absence of BQDs, DOX, BQD-Apt, and BQD-Apt-DOX, and \(OD_{treated}\) was obtained in the presence of BQDs, DOX, BQD-Apt, and BQD-Apt-DOX.
Figure S1. The fluorescence emission spectrum (Ex=405 nm) for BQDs solution under different reaction temperatures (A) and different times (B).

Figure S2. XRD of BQDs.
Figure S3. (A) Full XPS spectrum, (B) C1s XPS spectrum, (C) N1s XPS spectrum, and (D) O1s XPS spectrum of the obtained BQDs.

Figure S4. Effect of different pH values (A) and irradiation time of 365 nm UV light (B) on the fluorescence intensity of BQDs.
Figure S5. Fluorescence emission spectra for BQDs solution under different excitation wavelengths.

Figure S6. Fluorescent lifetime curve of BQDs.
Figure S7. UV-vis spectra of BQDs, free DOX, Apt, BQD-Apt, and BQD-Apt-DOX.

Figure S8. FTIR spectra of BQDs, Apt, and BQD-Apt complex.
Figure S9. (A) Loading efficiency study. (B) Percentage of DOX release from BQD-Apt-DOX versus time in PBS solution with different pH values.

Figure S10. Fluorescence emission spectra for BQD-Apt-DOX in PBS solution with different pH values (pH 5.5, 6.8, and 7.4) at 37 °C for 1 h (A), 6 h (B), 12 h (C), and 24 h (D), Ex=405 nm.
**Figure S11.** Confocal microscopy images of DOX in HeLa cells. HeLa cells were incubated with BQD-Apt-DOX for different times. The imaging of DOX was collected at 580-630 nm under the excitation of 488 nm.

**Figure S12.** Laser confocal fluorescence imaging of BQD-Apt-DOX-stained HepG2 cells for different incubation times. Excitation: 405 nm; Emission: 480-530 nm for green channel, 580-630 nm for red channel.
Figure S13. Laser confocal fluorescence imaging of BQD-Apt-DOX-stained 7702 cells for different incubation times. Excitation: 405 nm; Emission: 480-530 nm for green channel, 580-630 nm for red channel.