Electronic supplementary information

Fluorescent COFs with highly conjugated structure for drug visual loading and responsive release

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Section 1. Experimental details

Raw materials

TPA is made in the lab, and benzidine and trifluoroacetic anhydride were purchased from Shanghai McLean reagent co. LTD. Other chemicals used in our experiments were purchased from Sinopharm Chemical Reagent Co. Ltd. All the chemicals were of analytical grade and were used without further purification. Ultrapure water was used in our experiments.

1.1 Preparation of COF

The synthesis process of formyl-substitued triphenylamine was divided into two steps. First, the trifluoroacetyl-imidazole-triphenylamine intermediate (TPA-TIT) was synthesized: 2.0 g (8.2 mmol) TPA, 2.5 g (36.8 mmol) imidazole and 40 mL acetonitrile were added into a 100 mL four-mouth bottle and stirred for 30 min. Then

14.9 g (70.9 mmol) trifluoroacetic anhydride was added dropwise, the solution turns yellowish green, stir and heat for another 1.5 h. The reaction was terminated in ice water (80 mL), filtered and cleaned by cool water, stoved at 60 ^{o}C in vacuum. Yellow solid product was obtained, yield 7.6 g (98.32 %), purity: 84.70 %. Mp: 257 -259 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.37 (d, 6H, J = 8.5), 7.26 (s，3H), 7.05 (d, 6H, J = 8.4), 6.70 (s, 6H).

2.0 g (2.1 mmol) TPA-TIT, 60 mL tetrahydrofuran and 70 mL dilute hydrochloric acid (3.5 mol/L) were added into 250 mL four-port bottle, stirred and heated for 12 h. The reaction was terminated in ice water (100 mL). Extracted by tetrahydrofuran (30 mL×3), dried with anhydrous magnesium sulfate, and distilled under reduced pressure, the target product was obtained. Yield: 0.64 g (91.43%), purity: 87.50. The relevant NMR was placed in the supplementary material.^{1}H NMR (500 MHz, CDCl3) δ 9.95 (s, 1H), 7.85 (d, J = 8.7 Hz, 2H).

5.33 g (10 mmol) tri (4-formyl phenyl) amine, 10 mL tetrahydrofuran and 70 mL diluted hydrochloric acid (3.5 mL·L^{-1}) were added to a 250mL four-neck-flask and heated with reflux for 12 h. TLC(silica gel GF254 tablet developer) tested petroleum ether/ethyl acetate (5:1) reaction was completed, and then cooled to room temperature. The obtained sample was slowly poured 500 mL of deionized water, and 0.64 g of vacuum dried yellow solid was obtained after filtration. The yield was 91.43%.

1.2 drug loading

Approximately 1 mg of DOX was dissolved in 1 mL of water and slowly added to 4 mL of phosphate buffered saline (PBS) in which COF (4 mg) was dissolved. The mixture was stirred at room temperature for 24 hours and then centrifuged at 8000 rpm. Finally, the drug loaded sample was washed by PBS to remove unabsorbed DOX.

COF (400 mg) was immersed in PBS (4 mL) and various concentrations of DOX were added and stirred in dark for 24 h. Any unloaded DOX was removed by repeated wash and high speed centrifugation, and the COF loaded DOX washed using PBS and followed by freeze-dried for 24 h. UV-visible spectroscopy was used to measure the unbound DOX content in supermatant, and loaded DOX to COF was calculated based

on previously constructed methods. The drug loading efficiency (DLE) of the COF can be calculated using Equations (2).

1.3 Drug release

Cumulative release of DOX from COF+DOX was studied at two different pH conditions; pH 5 was comparable to intracellular pH of cancer cells and pH 7.4 was the extracellular pathophysical pH of normal cells. Briefly, 300 mg of COF+DOX was dispersed in 10 mL of PBS and sealed inside a dialysis bag (molecular cut off 50 kDa). It was then immersed in 200 mL of external buffer medium kept in a bowl. Dialysis was continued at 37 ^{o}C and drug release was monitored by UV-Vis by measuring the absorbance of the external buffer at 488 nm (against a standard solution).

Drug release was tested under different pH conditions (pH=7.4 and pH=5.0). COF+DOX samples were dissolved by ultrasound in PBS (10 mL, pH=7.4 and pH=5.0), and then put into dialysis bag, followed by shock release in corresponding PBS for 72 h at 37 ^{o}C. 4 mL dialysate was taken out at different times and the absorbance (λ_{ab}=488 nm) was measured to assess the DOX release rate. Meanwhile, 4 mL new PBS was added to maintain overall volume. The experiment was repeated three times and the average value was taken. The cumulative release rate can be calculated by Equation (3) and (4).

1.3 Visual drug loading

In order to determine the effect of adding different amounts of DOX on COF fluorescence, we prepared groups (4 mL per group) of DOX solution (DOX 20 μg/mL) and COF with gradually increased quality (120-600 μg) were added to the above solution. After all COF was dissolved by ultrasonication method, the solutions were stirred at a constant speed (20 rad/s) for 24 hours to ensure that DOX could be fully loaded. The solution of COF+DOX was firstly photographed under 365nm ultraviolet light and natural light. In addition, the fluorescence changes of COF were also

monitored by fluorospectrophotometer. Specifically, 100 μL COF solution (50 μg/mL) was added to 4 mL DOX solution each time, and the fluorescence intensity of COF was measured after shaking for 5 minutes. The concentration of COF in mixed solution increased from 0 μg/mL to 150 μg/mL.

1.4 Maximum drug loading and sustained release

The DOX was successfully loaded onto the COF by simply mixing with the DOX. The specific operation was to dissolve 3 mg of the sample in 10 mL of PBS solution, sonicated for 1 h to dissolve it evenly, and then add 1 mg of DOX. After being stirred for 24 hours in the dark, excess DOX was centrifuged several times through a high-speed centrifuge, and the supernatant was left to be tested for the UV verification load. The lower solid material was washed several times, and then vacuum freeze-dried to determine the peak of the ultraviolet curve at 480 nm measure the direct load. Drug loading capacity (DLC) and drug loading efficiency (DLE) could be calculated by the following formula:

$$\mathrm{DLC\,(mg/mg)} = \frac{M_D - M'_D}{M_F} \quad (1)$$

$$\mathrm{DLE\,(\%)} = \frac{M_D - M'_D}{M_D} \times 100\% \quad (2)$$

In the equations, Where M_D is the total amount of DOX added, M'_D is the amount of DOX without loading and M_F is the total amount of COF in COF+DOX added.

The dried DOX loaded samples were separately placed in a solution of PBS (pH=7.4 and pH=5.0), then transferred to a dialysis bag, immersed in a 200 mL PBS solution flask of the same pH, shaken at 37 ^{o}C for 24 hours. 4 mL of PBS in the cone bottle was taken every 4 hours during shaking, and replaced with 4 mL of fresh PBS. The extracted PBS was left to measure the UV data at 480 nm to observe the simulated release of DOX.

$$W_1\% = \frac{C_0V_0 - C_1V_1}{C_0V_0} \times 100\% \qquad (3)$$

$$W_2\% = \frac{C_{ti} \times V + \sum C_{ti} \times V_{ti}}{C_0 V_0 - C_1 V_1} \times 100\% \qquad (4)$$

Where W_1 is the drug loading rate, W_2 is the cumulative release rate, and C_0 is the concentration of the initially added DOX. V_0 is the volume of the initially added DOX, and C_1 is the concentration of the drug in the supernatant obtained by centrifugation after drug loading, V is the volume of the phosphate buffer, and V_1 is the volume of the supernatant obtained by centrifugation after the drug is loaded. When C_{ti} is concentration of the drug at a certain moment in the buffer solution taken out, V_{ti} is the volume of the buffer solution taken out at a certain moment.

1.5 In vitro cytotoxicity assay analyses

To test the toxicity of the drug delivery to the cells, we used MTT cell assay technology. A549 cells were cultured in a medium containing RPMI 1640 containing 10% by volume of inactivated calf serum and penicillin at 100 KU/L, and cultured at 37 ^oC with CO_2 (volume fraction 5%). The cells were cultured in a box and digested with 0.25% trypsin every 2-3 days, and passaged at 1:3-1:5 (the density of cells after passage was 1/3-1/5). After the prepared A549 cells were placed in a 96-well plate (5×10^3 cells/well), the samples, pure DOX, and sample with DOX were sequentially added according to five pre-designed concentration gradients. After 24 hours of culture, 15 mL (5.0 mg mL^{-1}) was added to each well. MTT solution, continue to culture for 4 hours, after aspirating the MTT in the well, add 100 μL of DMSO solution, gently shake the plate for 10 minutes to dissolve the crystallized substance, measure the absorbance at 488 nm on a microplate reader, and use a blank sample as a control, according to the formula Cell viability was calculated.

$$W_3\% = \frac{A - A_0}{A_0} \times 100\%$$

In the formula, W_3 is the cell survival rate, A is the absorbance of the test well, and A_0 is the absorbance of the control well.

1.6 Intracellular DOX uptake and fluorescence imaging

Cellular uptake of drugs was assessed by flow cytometry. Briefly, A549 cells were seeded in 2 mL DMEM medium in 6-well plates in a humidified 5% CO_2

atmosphere. After pre-incubation for 24 h, the old DMEM were replaced with fresh medium, and the cells were then freed with DOX and vectors. The samples were incubated together to ensure DOX concentrations of 1 mg/mL and 2.5 mg/mL, respectively. After incubation for 4 hours at constant temperature, the cells were washed three times with PBS and collected by trypsin-EDTA. 1.0 mL of PBS was added to each well, followed by centrifugation at 1000 rpm for 5 minutes. Subsequently, the supernatant was removed and 0.3 mL of PBS was added. A549 cells were seeded on pre-sterilized glass plates and the uptake of DOX in the cells was monitored by CLSM. The cells were pre-incubated for 24 h, and the free DOX and drug-loaded samples were added to a six-well plate. The DOX was kept at 2.5 mg/mL during the experiment. After constant temperature incubation for a different time, a certain amount of paraformaldehyde PBS solution was added dropwise, the cells were fixed for 20 minutes, and then the nuclei were stained with 4',6-diamidino-2-phenylindole. Finally, CLSM was used to observe the location of DOX within the cells.

The cytotoxicity of COF free DOX and COF+DOX against A549 cells was measured by standard MTT. After 5 minutes of shock, absorbance of cells at 570 nm in wells with different concentrations was measured by microplate reader. For the cell migration studies, we used the classical cell scratch method to measure the treated cells and the control cells. MSHOT software was used to measure the width of scratches under an inverted microscope to determine cell migration.

1.7 Cell migration experiment

Cancer cell line A549 was maintained following standard protocol. Migration was measured by wound healing assay, in which cells were grown to 80% confluence in 6-well plates, streaked with a sterile pipette tip, and allowed to recover in media. COF, DOX and COF+DOX solutions of 5 μg/mL and 10 μg/mL were added to different groups, respectively. After 24 h, plates were visualized under inverted microscope and migration determined by measuring wound width using the professional software.

Section 2.Figures S1–S10

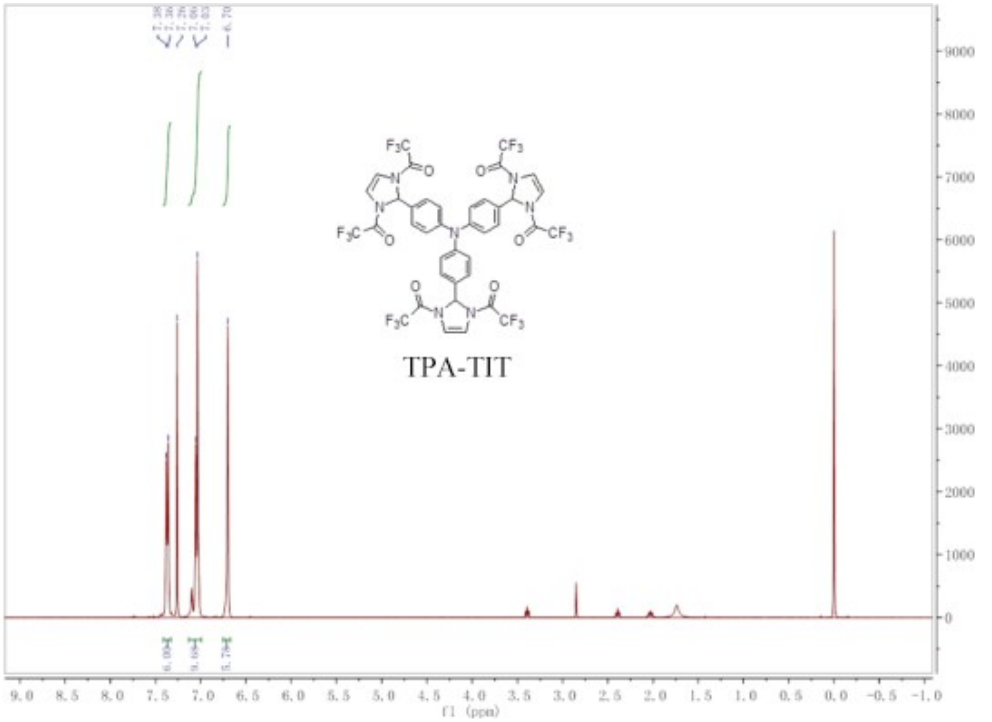

Fig. S1 TPA-TIT NMR image

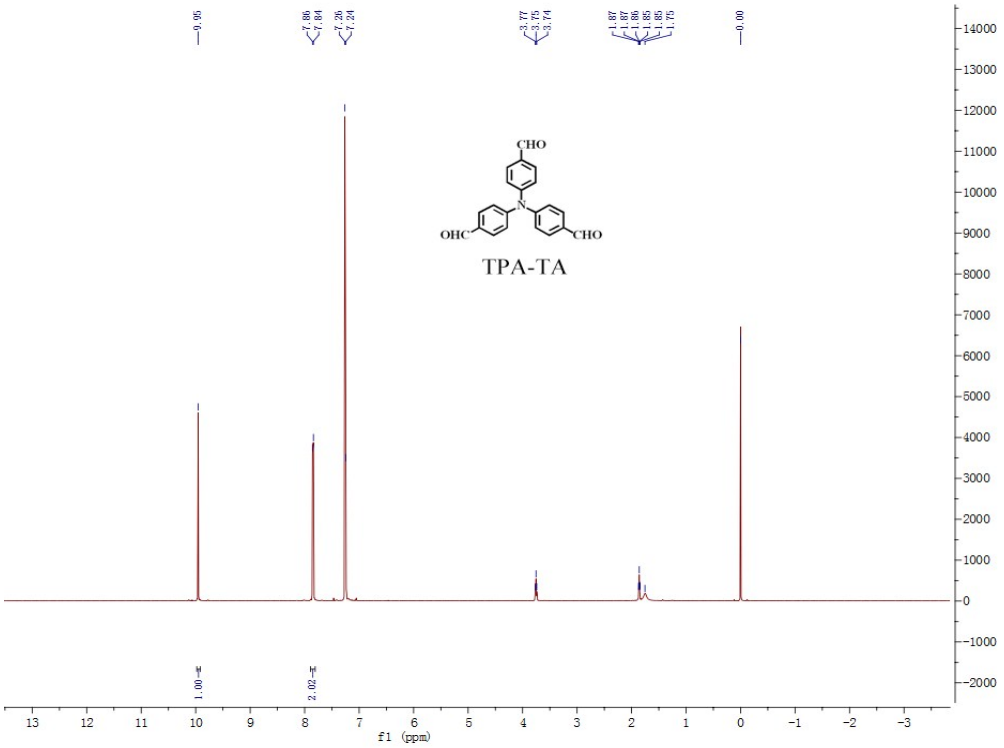

Fig. S2 TPA-TA NMR image

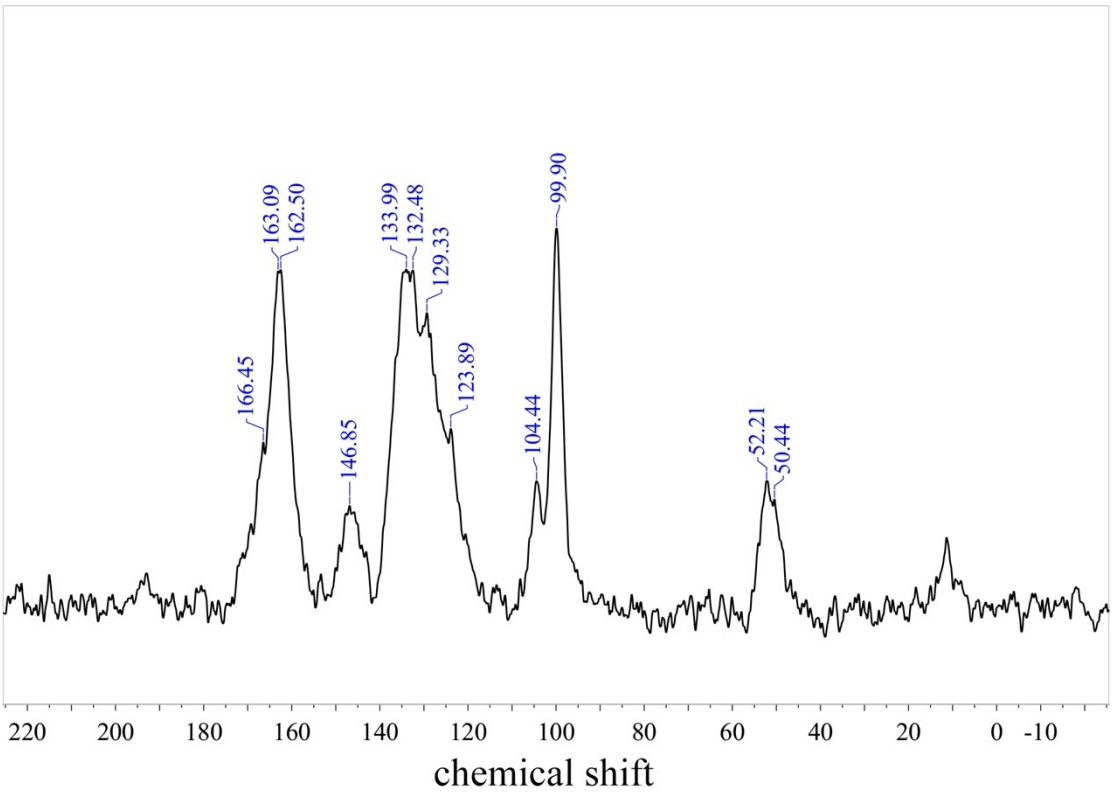

Fig. S3 ^{13}C CP MAS solid state NMR spectrogram of COF.

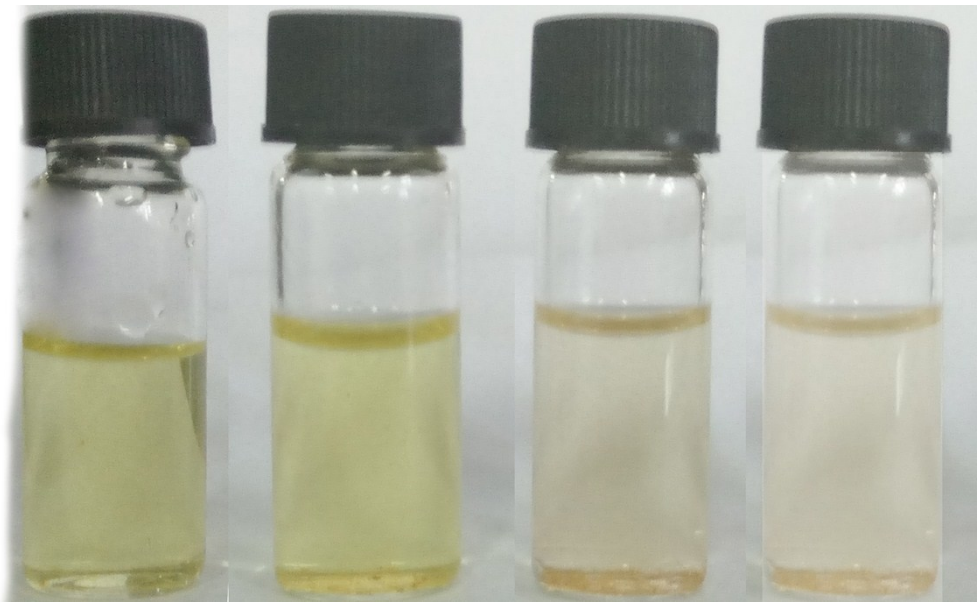

Fig. S4 Image of COF dissolution samples in DMSO, DMF, DAC and EAC.

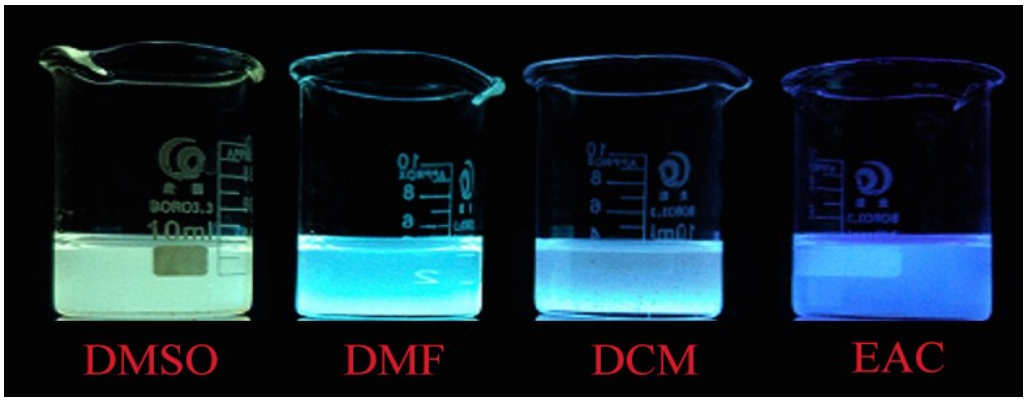

Fig. S5 PL images of COF dissolution in DMSO, DMF, DAC and EAC.

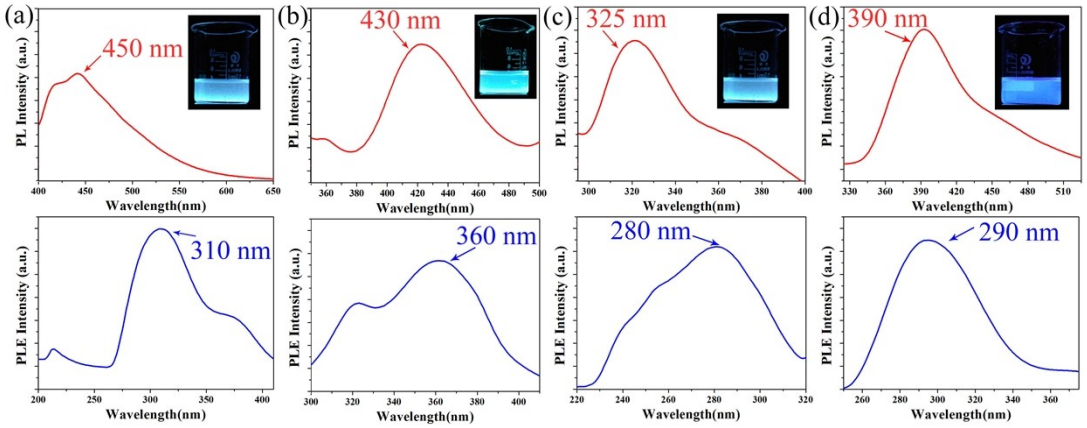

Fig. S6. The PL (top) and PLE (bottom) property of COF in (a) DMSO, (b) DMF, (c) DCM, and (d) EAC. Insets show corresponding PL images.

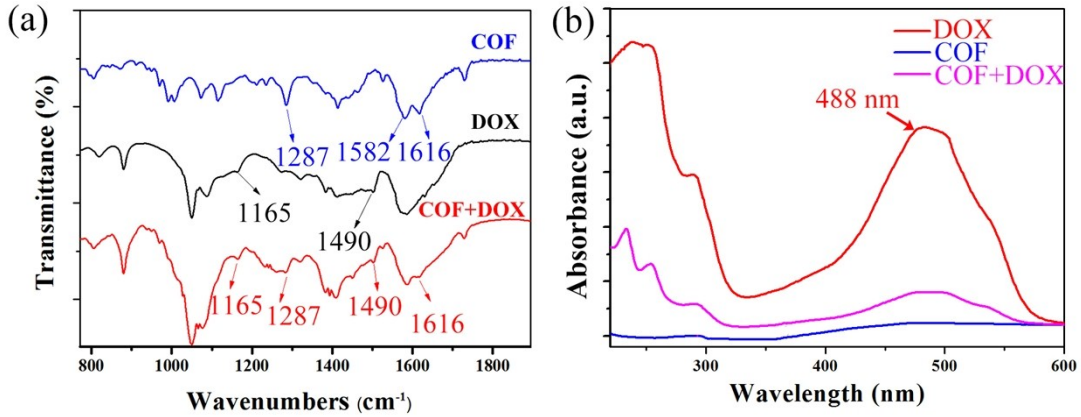

Fig. S7. (a) FTIR and (b) UV-vis data for DOX and COF+DOX.

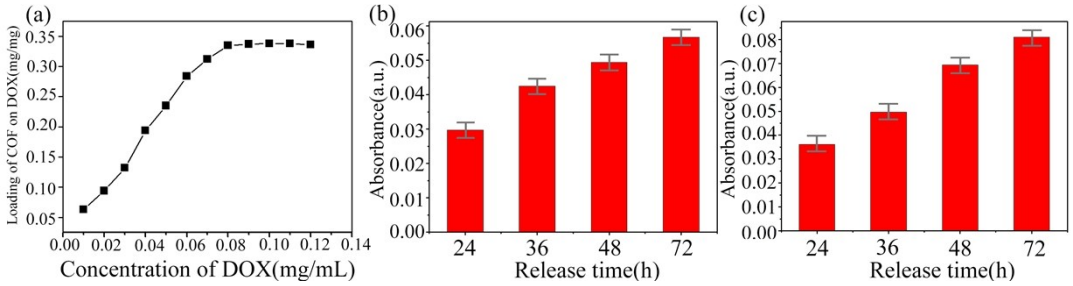

Fig. S8 (a) Drug loading capacity of COF. pH-responsive drug release performance of COF+DOX under (b) acid (pH=5.0) and (c) neutral conditions (pH=7.4).

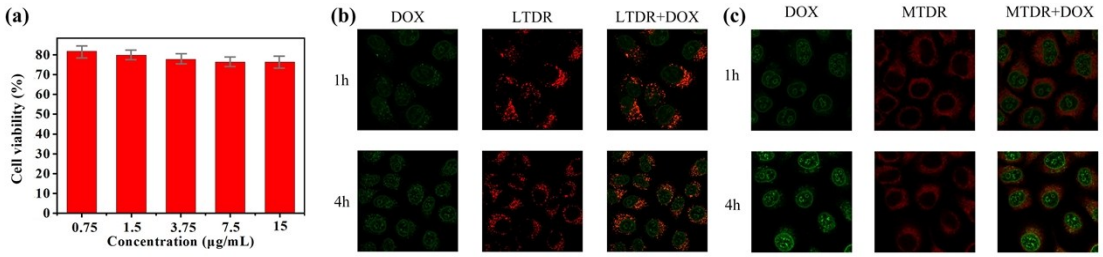

Fig.S9. (a) Cell viabilities of A549 cells incubated with different concentrations of COF (0.75-15 μg/mL) for 24 h.(b) and (c) CLSM images for A549 cells incubated with COF+DOX after 1 h and 4 h.

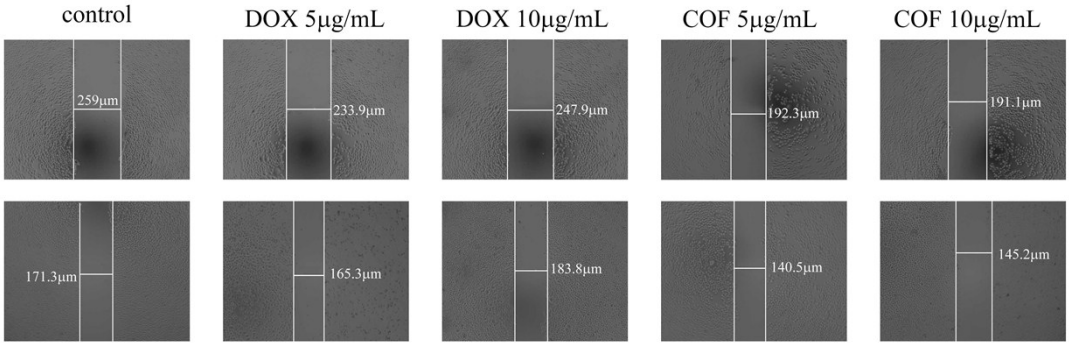

Fig.S10. Comparison of cellular migration between control, pure DOX and pure COF.