Supporting Information

Poly β-Cyclodextrin Inclusion-induced Formation of Two-Photon Fluorescent Nanomicells for Biomedical Imaging

Huijuan Yan[a], Leiliang He[a], Cheng Ma[a], Jishan Li*[a], Jinfeng Yang[b], Ronghua Yang*[a], and Weihong Tan[a]

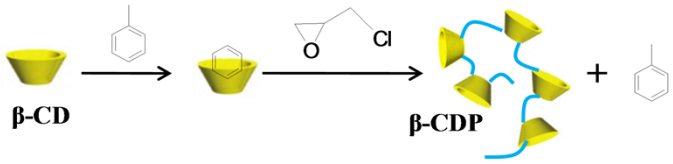
[a] *State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University,*
[b] *the Tumor Hospital of Xiangya School of Medicin Central South University, Changsha 410082, P. R. China*
E-mail: jishanli@hnu.edu.cn, yangrh@pku.edu.cn

Materials. 4-picoline, methyl iodide, piperidine, 4-(N, N-dimethylamino)-benzaldehyde and β-cyclodextrin (βCD) were purchased from Sinopharm Chemical Reagent Co. (China). Methyl thiazolyl tetrazolium (MTT) was purchased from Sigma-Aldrich. Cyclo(Arg-Gly-Asp-Phe-Lys(mpa)) pepetide (c(RGDyK)) conjugated adamantine (denoted as Ad-RGD, purity: 98.5%) was purchased from ChinaPeptides Co., Ltd. (Shanghai, China). The MCF-7 (human breast cancer, low $\alpha_v\beta_3$ integrin expression) and HeLa (cervical cancer, high integrin $\alpha_v\beta_3$ expression) cell lines were provided by the Biomedical Engineering Center of Hunan University (China). Cervical cancer tissue slices and normal cervical tissue slices were provided by the Hunan Provincial Tumor Hospital, Central South University (China). The study was approved by the Ethics Committee of Hunan Provincial Tumor Hospital. 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, USA) and had an electric resistance >18.3 MΩ. All experiments were carried out at room temperature.

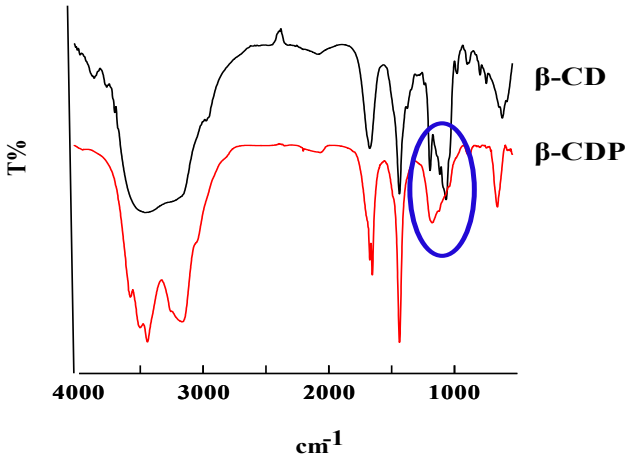
Apparatus. Nuclear magnetic resonance (NMR) spectra were recorded at 400 MHz on an Invoa 400 spectrometer. Chemical shifts (δ) are reported in parts per million relative to tetramethylsilane using the residual solvent peak as a reference standard. Coupling constants (J) are reported in hertz. Mass spectra

(MS) were obtained on LCQ/Advantage HPLC-Mass spectrotometer (Thermo Finnigan). In order to observe morphology of the DEASPI/βCDP TPA nanomicells and the success of the nanomicell formation procedure, Transmission Electron Microscopy (TEM) (JEOL JEM-3010), dynamic light scattering (Malvern Zetasizer 3000HS) and circular dichroism spectra (MOS 500 spectropolarimeter, Bio-Logic) were employed. UV-vis absorption spectra were obtained on a Hitachi U-4100 UV/Vis spectrometer (Kyoto, Japan) using a quartz cuvette having 1 cm path length. One-photon excitation (OPE) fluorescence spectra were obtained on a Hitachi F-7000 fluorescence spectrofluorimeter equipped with a 1000 W Xenon lamp (Kyoto, Japan). Two-photon excitation (TPE) 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.). Two-photon fluorescence images of cells and tissues were obtained using an Olympus FV1000-MPE multiphoton laser scanning confocal microscope (Japan). The pH values were calibrated with a model 868 pH meter (Orion).

Synthesis of β-Cyclodextrin Polymer (βCDP). βCDP was synthesized according to the literature reported previously (Scheme S1).[1] Briefly, 5.0 g (4.4 mmol) of βCD is dissolved by stirring for at least 2 h in 7.5 mL of a 15 wt% aqueous sodium hydroxide solution at 35°C. Subsequently, 1.0 mL (4.4 mmol) of toluene was added to synthesize βCDP. After an additional 2 h of stirring at 35 °C, the accordant amount of epichlorohydrin was added to the mixture. After 3 h of stirring, the solution was added to 100 mL of isopropanol and the precipitate was filtered. The raw product was dissolved in water, neutralized with diluted hydrochloric acid and dialyzed for 7 days (MWCO 8000). The product was isolated via lyophilization. Yield: Maximum 50% βCDP by application of 5 mol% epichlorohydrin. The FTIR spectra of βCD and βCDP show that most absorption bands of βCD are still present in the spectrum of βCDP and a new absorption band at 2099.5 cm^{-1} attributed to the $–(CH_2)_n–$ units can be observed in the spectrum of βCDP. Moreover, the absorption band of stretching vibration of C-O-C at 1070~1160 cm^{-1} is broadened in the spectrum of βCDP due to the cross-linking reaction of βCD. According to the literature, the polymer molecular weight is about 96 kD, containing ca 100 βCD units in one βCDP.

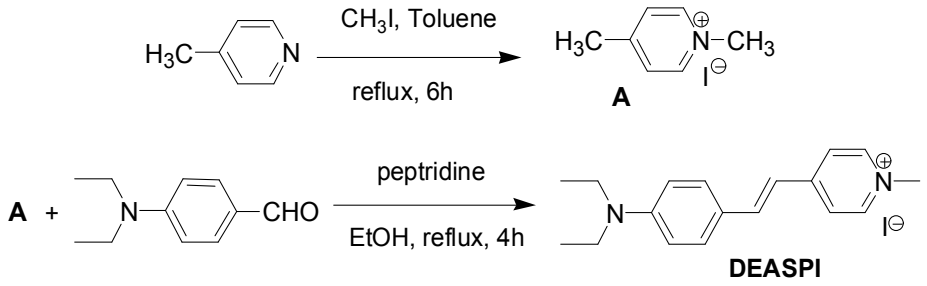


Scheme S1. Synthetic routs for βCDP.

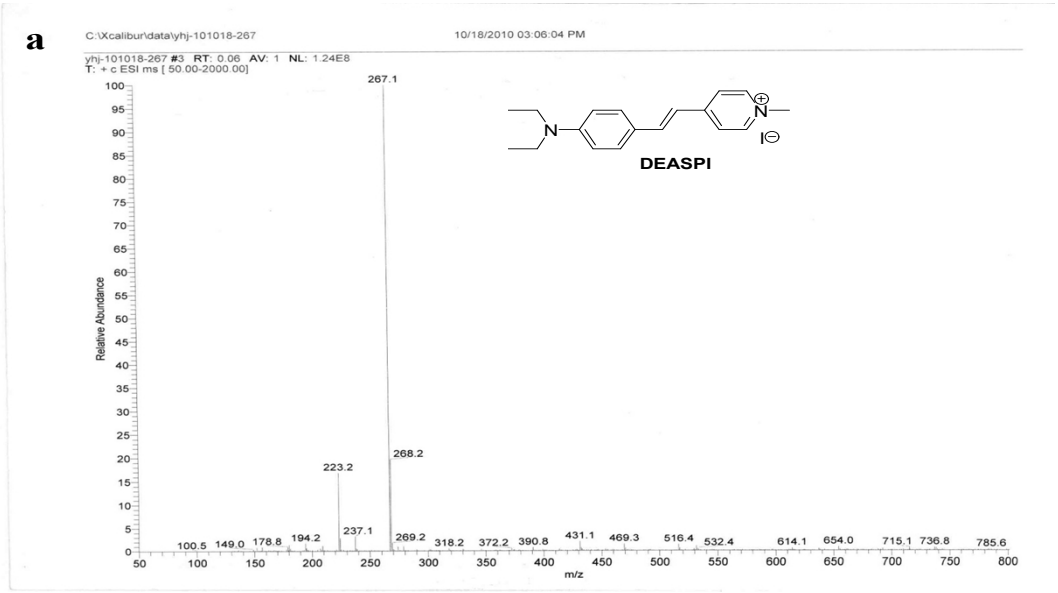


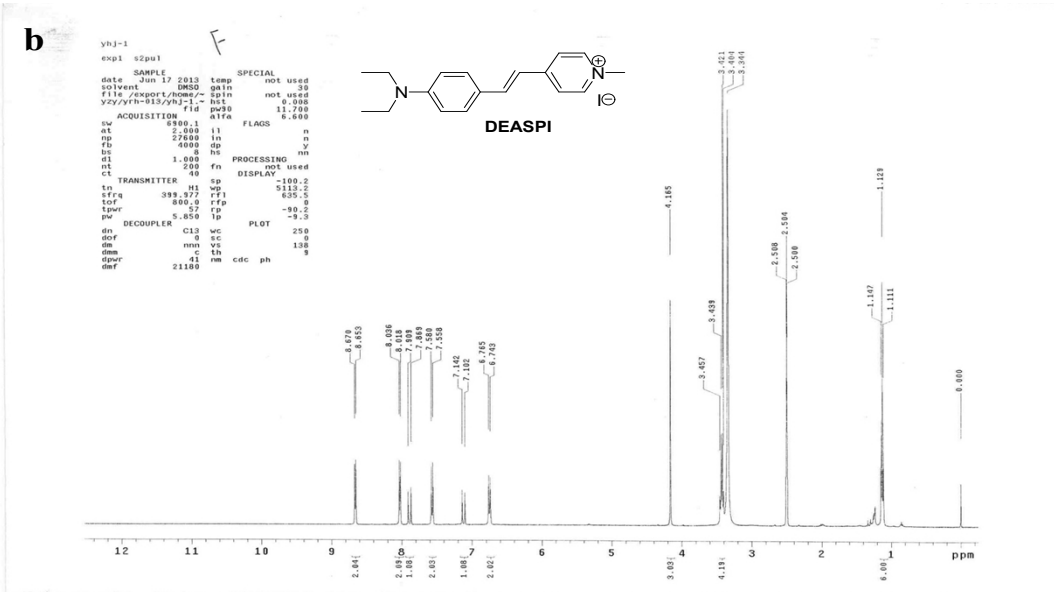
IR spectra of βCD and βCDP.

Synthesis of Trans-4-[p-(N,N-diethylamino)styryl]-N-methyl-pyridinium iodide (DEASPI). The synthetic rout for DEASPI was according to the above scheme (Scheme S2).[2] First, using a three-neck flask fitted with a stirrer, thermometer, and condenser, 4-picoline (1.0 equiv.) and methyl iodide (1.1 equiv.) were mixed in toluene. The solution was stirred at room temperature for 4 h and then refluxed for 2 h. After cooling, the solution was filtered and the solid was washed with ethyl ether. The pale yellow solid powder (1: 4-methyl-N-methyl-pyridinium iodide) was dried under vacuum, yield: 4.32 g (60%); ESI-MS, m/z: 108.0 ($[M-I]^+$). Then a mixture of 1 (1.0 equiv.) and 4-(N, N-dimethylamino)-benzaldehyde (1.0 equiv.) in dried ethanol was treated with piperidine and was refluxed for 4 h. The resulting suspension was cooled. Then the suspension was filtered and concentrated. The residue was purified by column chromatography on silica gel using ethanol as eluent to yield DEASPI (70%). ^{1}H NMR (400Hz, DMSO-d_6) δ (ppm): d: 8.67 (2H, d, J 6.84Hz), 8.04 (2H, d, J 6.84 Hz), 7.91 (1H, d, J 15.61Hz), 7.58 (2H, d, J 8.79Hz), 7.12 (1H, d, J 15.63Hz), 6.75 (2H, d, J 8.80Hz), 4.18 (3H,s), 3.43 (4H, q, 6.84), 1.13 (6H, 6.84); IR (KBr) ν/cm^{-1}: 3413.88, 2970.12, 826.64; ESI-MS, m/z: 267.1 ($[M-I]^+$).



Scheme S2 Synthetic routs for DEASPI.





(a) ESI-MS of and (b) ^{1}H NMR of DEASPI.

Preparation of DEASPI/βCDP Nanomicell. The preparation of βCDP-based TP fluorescent nanomicell was typically conducted as follows. βCDP was first suspended in 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) at an initial concentration of 5 mg/mL. Then 1 mM DEASPI was added into the above solution at a final concentration of 100 μM and the mixture was stirred for 1 h. The obtained DEASPI/βCDP nanomicells was stored at 4 ^{0}C before analysis.

Preparation of RGD-functionalized DEASPI/βCDP Nanomicells (DEASPI/βCDP@RGD). For the preparation of DEASPI/βCDP@RGD nanomicells, Ad-RGD was added into the DEASPI/βCDP nanomicell solution at a final concentration of 50 μM and incubated for 2 h at room temperature to guarantee the anchoring of c(RGDyK) peptide on the surface of DEASPI/β-CDP nanomicell. Then the mixture was filtrated with an Amicon YM-10 filter to get rid of the free Ad-RGD, and the obtained DEASPI/βCDP@RGD nanomicell was resuspended in PBS buffer and stored at 4 ^{0}C before use. TEM image of the obtained DEASPI/βCDP@RGD nanomicell shows that its morphology is still spherical shape (Figure S4), and the Zeta potential of the particles changed from -11.6 mV of DEASPI/βCDP to -1.71 mV of DEASPI/βCDP@RGD owing to positive charges of the c(RGDyK) peptide chain in aqueous phase, indicating that the efficient immobilization of Ad-RGD on DEASPI/βCDP nanomicell's surface and the DEASPI/βCDP@RGD nanomicell was obtained in deed.

Sample Preparation for TEM Images. Samples for TEM analysis were prepared by pipetting 10 μL of the colloidal solutions onto the carbon membrane support surfaces, and the excess solvent were blotted with the filter paper but not be dried. On this condition, the samples were measured in aqueous environments and could avoid the deformation of the particles during the sample preparation.

Cell Culture. The MCF-7 cells were grown in MEM (modified Eagle's medium) supplemented with 10% FBS (fetal bovine serum) and 1% insulin (10 mL: 400 U), and the HeLa cells were grown in MEM supplemented with 10% FBS. Cultures were maintained at 37 °C under a humidified atmosphere containing 5% CO_2.

Cytotoxicity Assay. The cellular cytotoxicity of DEASPI/βCDP nanomicells towards HeLa and MCF-7 cells as the model was evaluated using the standard cell viability assay — the MTT assay.[3] HeLa and MCF-7 cells were seeded into a 96-well plate at a concentration of 4×10^3 cells/well in 100 μL of MEM medium with 10% FBS. Plates were maintained at 37 °C in a 5% CO_2 95% air incubator for 24 h. After the original medium was removed, the HeLa and MCF-7 cells were incubated with DEASPI/βCDP nanomicells, βCDP and DEASPI with different concentration ([DEASPI] = 0, 5, 10, 20, and 50 μM; [βCDP] = 0, 200, 500, 1000, and 2500 μg/mL, respectively). The cells incubated with the culture medium only were served as the controls. The cells were washed with PBS for three times and then 100 μL MTT solution (0.5 mg/mL in PBS) was added to each well. After addition of DMSO (150 μL/well), the assay plate was allowed to shake at room temperature for 10 min. The spectrophotometrical absorbance of the samples was measured by using a Tecan microplate (ELISA) reader. The cell viability was calculated based on measuring the UV-vis absorption at 570 nm using the following equation, where OD570 represents the optical density.[4,5]

$$\textbf{Cell viability} = [OD_{570(sample)} - OD_{570(blank)}] / [OD_{570(control)} - OD_{570(blank))}] \qquad (1)$$

Living Cell Imaging. For cell imaging experiments, cells were seeded in 24-well 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 PBS. Then 150 μL of DEASPI/βCDP or DEASPI/βCDP@RGD (100 μM, calculated by dye's concentration) was added into each well, followed by incubation for 30 min at 37 °C. For blocking assay, the cells were first incubated with excess c(RGDyK) (10 μM) for 1 h at 37 °C prior to DEASPI/βCDP@RGD treatment. Finally, the treated cells were washed three times with PBS to remove unbounded nanomicells, and two-photon confocal fluorescence imaging of the cells was observed under an Olympus FV1000-MPE multiphoton laser scanning confocal microscope, with a mode-locked titanium-sapphire laser source set at wavelength 840 nm.

Imaging of Tissue Slice. The cervical tumor tissues and the normal cervical tissues were cryosectioned at -20 °C into slices of 8-μM thickness, blocked with 1:10 bovine serum for 30 min, stained with 0.3 mg/mL DEASPI/βCDP@RGD or DEASPI/βCDP for 30 min at 37 ^{0}C, washed three times with

phosphate buffered saline (PBS), and examined under an Olympus FV1000-MPE multiphoton laser scanning confocal microscope, with a mode-locked titanium-sapphire laser source set at wavelength 840 nm.

Deep Tissue Imaging. A 2.0 mm-thick cervical tumor tissue slice was incubated with 0.3 mg/mL DEASPI/βCDP@RGD in 1:10 bovine serum-contained PBS for 30 min at 37 ^{0}C. After washing with PBS to remove the remaining probe, two-photon confocal fluorescence imaging and the 3D two-photon confocal fluorescence images accumulated along the *z*-direction at depth of 0-500 μm with 60 × magnification of this treated tumor tissue were observed under an Olympus FV1000-MPE multiphoton laser scanning confocal microscope, with a mode-locked titanium-sapphire laser source set at wavelength 840 nm.

Measurements of One-photon Quantum Yields and Two-Photon Absorbance Cross Section. The one-photon quantum yields (QY) of samples were estimated using Rhodamine 6G (literature quantum yield: Φ_F = 0.95 in ultrapure water) as a reference standard (Table S1), which was freshly prepared to reduce the measurement error.[6] The quantum yield Φ as a function solvent polarity is calculated using the following equation:

$$\Phi_F = \Phi_{F,\mathrm{cal}} \cdot \frac{S}{S_{cal}} \cdot \frac{A_{cal}}{A} \cdot \frac{n^2}{n^2_{cal}} \qquad (2)$$

Where $\mathbf{\Phi_F}$ is the quantum yield, **S** is the areas' integral values of the corrected fluorescence spectra, **A** stands for the absorbance and **n** is refractive index. The subscript **cal** and no denote the standard and sample, respectively.

The two-photon absorption (TPA) cross sections (δ) of samples (in the wavelength range of 800-960 nm) in neutral conditions were determined using TPE method with femtosecond Ti-sapphire laser pulses described in previous literature (Table S1).[7] TP dye (10 μM) was dissolved in PBS buffer (pH 7.4), and the TPE fluorescence emission intensity was measured in the emission range 520-650 nm under excitation at 800-960 nm using Rhodamine 6G as the reference, whose TP properties have been well-characterized in the previous literature.[8] Intensities of TPE fluorescence emission of the reference and the

samples emitted at the same excitation wavelength were determined. The TPA cross section was calculated as the following equation:[9,10]

$$\delta_S = \frac{S_S}{S_R} \cdot \left[\frac{\Phi_R \cdot C_R \cdot n_S}{\Phi_S \cdot C_S \cdot n_R} \right] \delta_R \qquad (3)$$

Where subscript *S* and *R* denote the sample and the reference, respectively. ***S*** represents the intensity of TPE fluorescence emission, ***Φ*** is the fluorescence quantum yield, ***C*** denotes the concentration, and ***n*** represents the refractive index of the solvents.

References

1 C. Koopmans and H. Ritter, *Macromolecules*. 2008, **41**, 7418-7422.

2 G. Y. Zhou, X. M. Wang, D. Wang, C. Wang, X. Zhao, Z. S. Shao and M. H. Jing, *Mater. Sci.Technol.* 2001, **17**, s139-s148.

3 (a) T. Mosmann, *J. Immunol. Methods.* 1983, **65**, 55-63; (b) Y. B. Liu, D. A. Peterson, H. Kimura and D. Schubert, *J. Neurochem*. 1997, **69**, 581-593.

4 (a) T. Mosmann, *J. Immunol. Methods.* 1983, **65**, 55-63; (b) Y. B. Liu, D. A. Peterson, H. Kimura and D. Schubert, *J. Neurochem.* 1997, **69**, 581-593.

5 J. Tang, B. Kong, H. Wu, M. Xu, Y.C. Wang, Y. L. Wang, D.Y. Zhao and G. F. Zheng, *Adv. Mater.* 2013, **25**, 6569-6574.

6 J. N. Demasa and G. A. Crosby, *J. Phys. Chem.* 1971, **75**, 991-1024.

7 S. K. Lee, W. J. Yang, J. J. Choi, C. H. Kim, S. J. Jeon and B. R. Cho, *Org. Lett.* 2005, **7**, 323-326.

8 N. S. Makarov, M. Drobizhev and A. Rebane, *Opt. Express.* 2008, **16**, 4029-4047.

9 C. Xu and W. W. Webb, *J. Opt. Soc. Am. B.* 1996, **13**, 481-491.

10 M. Rumi, J. E. Ehrlich, A. A. Heikal, J. W. Perry, S. Barlow, Z. Hu, D. McCord-Maughon, T. C. Parker, H. Rockel, S. Thayumanavan, S. R. Marder, D. Beljonne and J. L. Bredas, *J. Am. Chem. Soc.* 2000, **122**, 9500-9510.

Table S1. Photophysical Data for DEASPI, DEASPI/βCD and DEASPI/βCDP in buffer.

Probe	$\lambda_{max}/\lambda_{fl,max}$[a]	$\varepsilon \times 10^4$ [b]	ϕ [c]	δ [d] [GM]	$\delta\phi$ [e] [GM]	K [f] [M^{-1}]	log K [g]
DEASPI	470/575	1.97	0.0234	44.5698	1.0429	—	—
DEASPI/βCD	470/575	1.86	0.1058	81.4855	8.6212	1.420×10^3	3.152
DEASPI/βCDP	470/575	1.69	0.4707	94.5394	44.4997	4.462×10^3	3.650

[a] λ_{max} values of the one-photon absorption and emission spectra in nm; [b] Molar absorbance; [c] Fluorescence quantum yield; [d] TPA cross section (Rh 6G in methanol at 840 nm as reference, DEASPI, DEASPI/βCD and DEASPI/βCDP in 20 mM PBS at 840 nm) in 10^{-50} cm^4 s $photon^{-1}$ (GM), the experimental uncertainty is of the order of 5-15%; [e] TPA action cross sections in GM; [f] Binding constant of host-guest interaction; [g] The logarithm of the binding constants.

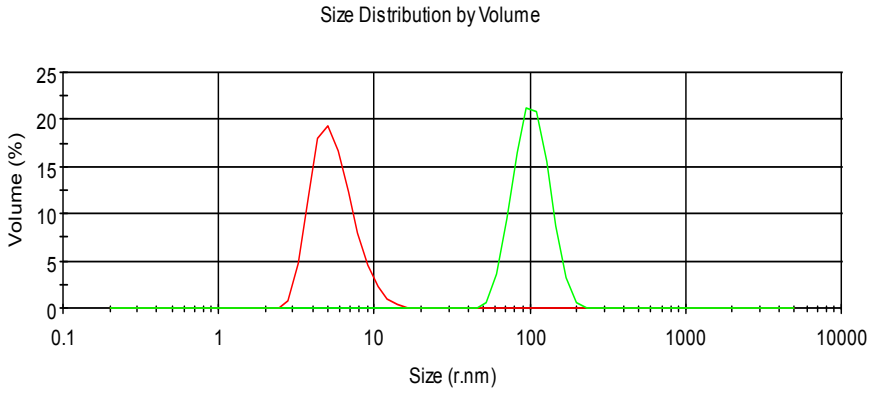


Fig. S1 DLS datas of βCDP (red line) and DEASPI/βCDP nanomicell (green line).

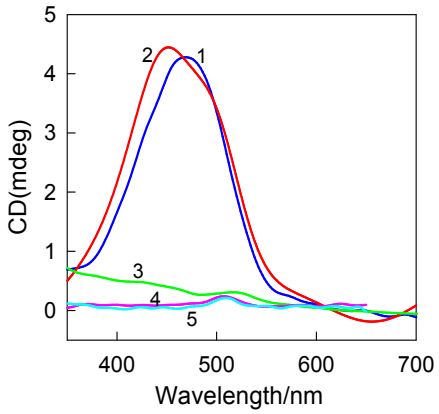


Fig. S2 Circular dichroism spectra of DEASPI/βCD (curve 1: 1.0 mM DEASPI and 5.0 mg/mL βCD), DEASPI/βCDP (curve 2: 1.0 mM DEASPI and 5.0 mg/mL βCDP), DEASPI (1.0 mM, curve 3), βCD (5.0 mg/mL, curve 4) and βCDP (5.0 mg/mL, curve 5).

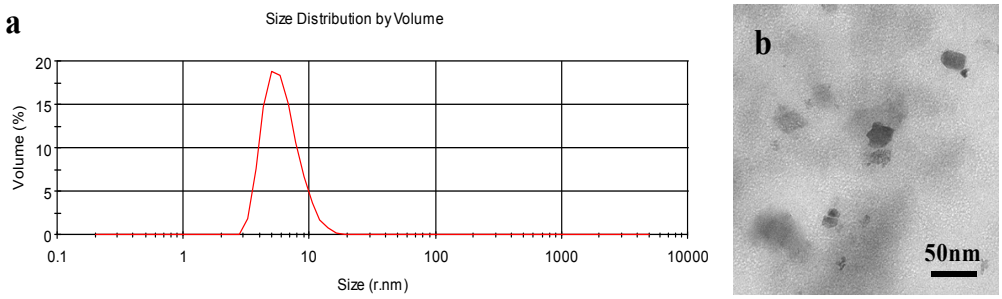


Fig. S3 DLS data (a) andTEM image (b) of the mixture of βCDP, adamantane and DEASPI.

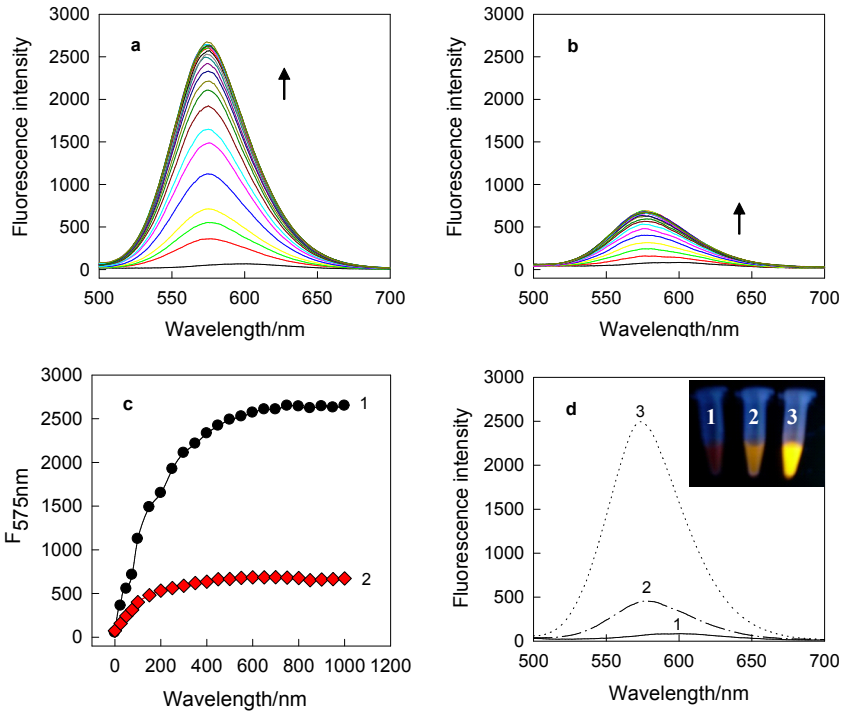


Fig. S4 Fluorescence emission spectra of DEASPI solution (10 μM) in the presence of different concentrations of βCDP (a) or βCD (b). The arrows indicate the signal changes as increases in the concentration of βCDP or βCD (0, 25, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000 μg/mL); (c) Fluorescence intensity at 575 nm of DEASPI versus the concentration of βCDP (curve 1) and βCD (curve 2) according to 'a' and 'b', respectively; (d) One-photon excitation fluorescence emission spectra and photographs (inset) of the DEASPI/βCDP nanomicells under UV irradiation. 1: 10 μM of DEASPI; 2: 10 μM of DEASPI + 0.5 mg/mL of βCD; 3: 10 μM of DEASPI + 0.5 mg/mL of βCDP. λ_{ex} = 470 nm.

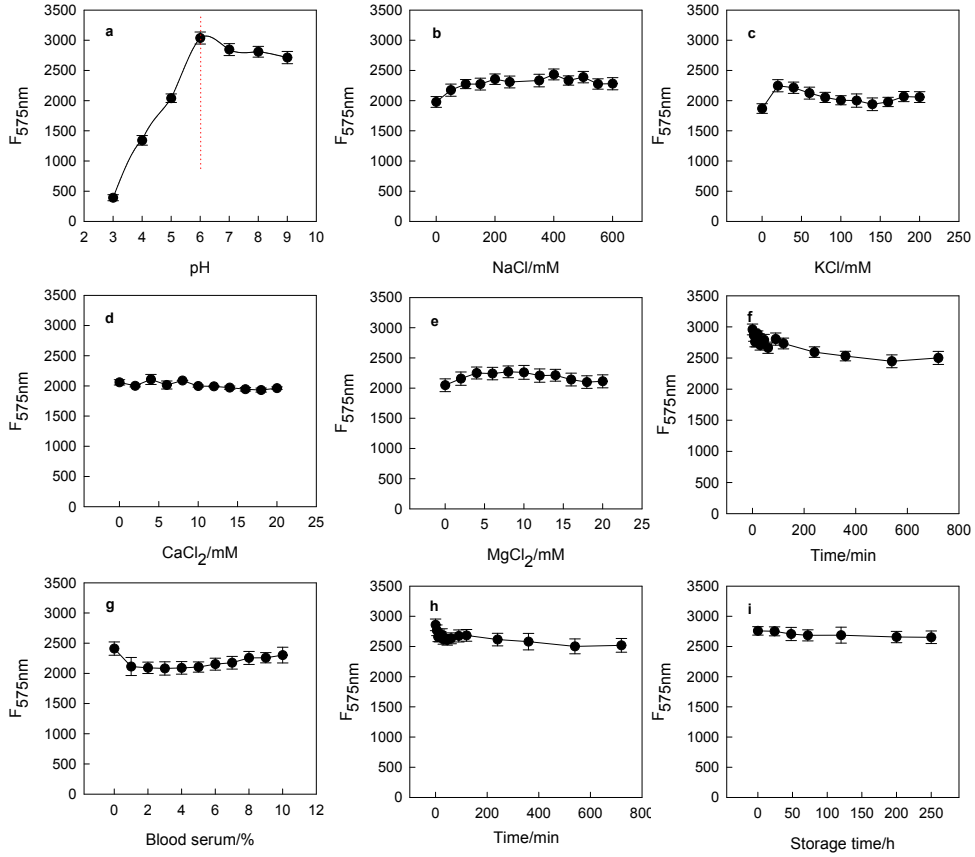


Fig. S5 Effects of different conditions on the fluorescence emission intensity of the DEASPI/βCDP nanomicell in aqueous solution (a-e) and the stability of DEASPI/βCDP nanomicell in PBS buffer (f), blood serum-contained solution (g) or HeLa cell lysate-contained solution (h). The storage stability of DEASPI/βCDP nanomicell (i). Fluorescence emission intensity was recorded at 575 nm with an excitation wavelength of 470 nm.

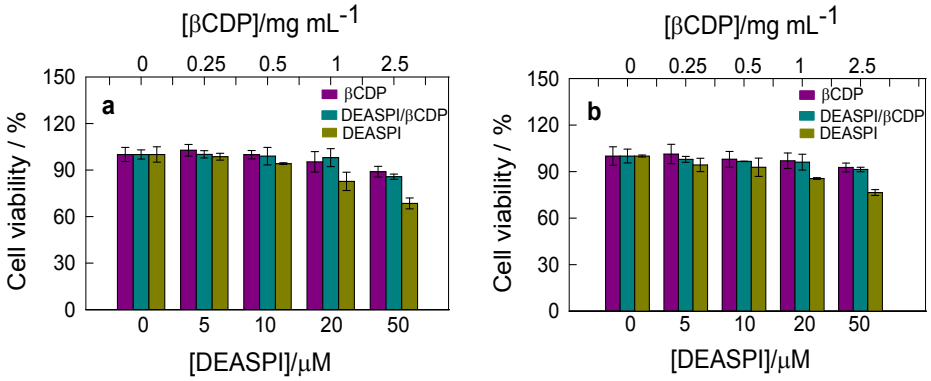


Fig. S6 Cell viability values (%) estimated by MTT proliferation. (a) HeLa cells or (b) MCF-7 cells were incubated with 0−2.5 mg/mL βCDP (DK pink), DEASPI/βCDP (DK cyan) and DEASPI (DK yellow) at 37 ^{0}C for 24 h. Cells without any addition were taken as the control experiment, and the viability was set

as 100%. The final reported data were expressed as a percentage of the control (mean standard deviation). Three independent experiments containing duplicates were performed.

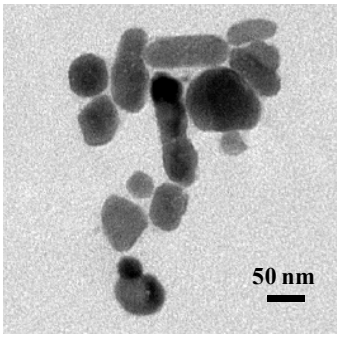


Fig. S7 TEM of the DEASPI/βCDP@RGD nanoprobe.

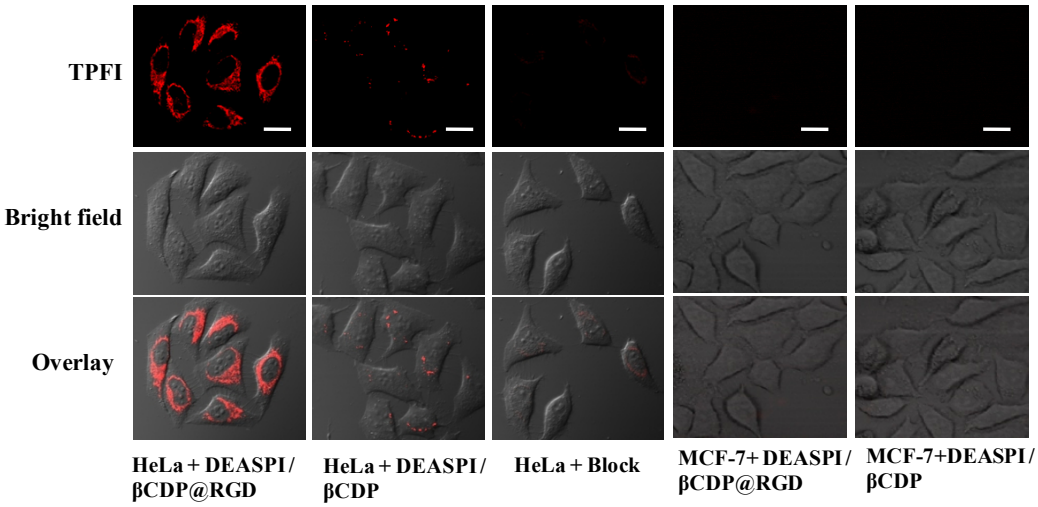


Fig. S8 Confocal two-photon fluorescence images of HeLa cells and MCF-7 cells incubated with DEASPI/βCDP@RGD or DEASPI/βCDP. For "HeLa + Block", 10 μM of c(RGDyK) was first used to treat the cells before addition of DEASPI/βCDP@RGD. Filter set: excitation wavelength is 840 nm and emission is 575 nm. Scale bar is 20 μm.

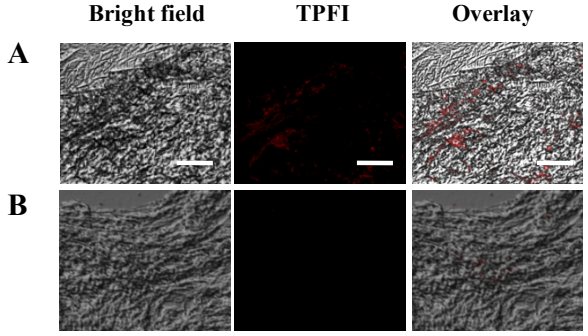


Fig. S9 TPE fluorescence images of frozen cervical cancer tumor tissue slice (A) and frozen normal cervical tissue slice (B) stained by DEASPI/βCDP. Scale bars: 40 μm.