# Supplementary Information

# Gradient-Hypoxia Responsive Nanocarrier Facilitates Enhanced Tumor Penetration and Improved Anti-tumor Efficacy

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1. **Characterization.** $^1$H NMR of was recorded on a 400 M NMR spectrometer (Bruker 400M, Germany) Dynamic light scattering (DLS) experiments were performed on a laser light scattering spectrometer (BI-200SM) equipped with a digital correlator (BI-9000AT) at 636 nm at a 90 ° scatter angle with required temperatures. Transmission electron microscopy (TEM) measurements were performed on Philips T20ST electron microscope at an acceleration voltage of 100 kV. The zeta potentials were measured on a Brookhaven ZetaPALS (Brookhaven Instrument, USA). The hypoxia-responsive properties of copolymers and micelles were studied by determining the transmittance using a UV−vis spectrophotometer (Purkinje General, China). The fluorescence intensity of doxorubicin was performed on a fluorescence spectrophotometer (F-7000, Hitachi, Japan). The fluorescence observation of cells was conducted by confocal laser scanning microscope (CLSM) (TCS SP8, Leica, Germany). Flow cytometry analysis was conducted by BD Calibur flow cytometry (BD Co., USA). The cytotoxicity experiments were detected by microplate reader (Labsystem, Multiskan, Ascent, Finland). The animal studies were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (Tianjin, revised in June 2004).

2. **Materials.** Methoxypolyethylene glycol ($CH_3O$-$PEG_{5k}$-OH) was purchased from Sigma-Aldrich. Tert-butoxycarbonyl amino-hydroxy poly(ethylene glycol) (Boc-NH-$PEG_{5k}$-OH) and $N_6$-Carbobenzoxy-L-lysine N-Carboxyanhydride (Lys-NCA; 98%) were obtained from Shanghai Yare Biotech (Shanghai, China). The above materials were dried under vacuum before use. ε-Caprolactone (ε-CL, 99%) and hydrogen bromide (33% w/w in acetic acid) were obtained from Alfa Aesar. 2-(N-tert-butoxycarbonylamino) ethanol (98%), stannous octoate ($Sn(Oct)_2$, 95%), 4-nitrobenzyl chloroformate (NBCF, 98%), trifluoroacetic acid ($CF_3COOH$, 99%) and N,N-Diisopropylethylamine (DIPEA, 98%) were purchased from J&K. Sulfo-cyanine 5 NHS ester was purchased from Lumiprobe Co. (Florida,

USA). Doxorubincin-HCl was purchased from Jingyan chemical Ltd. (Guangdong, China). The above materials were used as received. Dulbecco's modified eagle medium (DMEM) and other cell culture supplies were purchased from Gibco (Gibco Corporation, Grand Island, NY, USA) and used for MDA-MB-231 cell culture. The AneroPack-Anaero and AnaeroPack-MicroAero were purchased from Mitsubishi (Mitsubishi Corporation, Japan) and used to form an appropriate anaerobic (0.1% $O_2$) and micro-aerobic (8-9% $O_2$) culture environment, respectively. The Spraque Dawley rats were ordered from Beijing Vital River Laboratory Animal Technology Co., (Beijing, China).

## 3. Synthesis of block copolymers

### 3.1 Synthesis of PCL-*b*-PEG

The synthesis progress of PCL-*b*-PEG was shown in Fig. S1. The detailed experimental procedures referred the previous reports.[1] Briefly, $CH_3O$-$PEG_{114}$-OH (5 g, 1.0 mmol), *ε*-caprolactone (5 g, 87.8 mmol), 30 μL $Sn(Oct)_2$ and 15 mL toluene were added into the sealed tube. After three times of freeze-degas-thaw, the reaction was carried out at 110 °C for 2 days. The reaction solution was precipitated with cold diethyl ether, then filtered and dried in vacuum. According to the $^1$H NMR, the polymer was determined as HO-$PCL_{40}$-*b*-$PEG_{114}$.

### 3.2 Synthesis of PCL-*b*-PLL

The synthesis route of PCL-*b*-PLL was shown in Fig. S2. The detailed experimental procedures referred the previous reports.[2] PCL-*b*-PLL(Z) was obtained through ring opening polymerization (ROP) of N6-Carbobenzoxy-L-lysine N-Carboxyanhydride (Lys-NCA), followed by deprotection of PCL-*b*-PLL(Z) to obtain PCL-*b*-PLL.

Briefly, $PCL_{40}$-$NH_2$ (2.0 g, 4 mmol), N6-Carbobenzoxy-L-lysine N-Carboxyanhydride (Lys-NCA) (2.0 g, 7.14 mmol) and 15 mL DMF were added into the Schlenk tube. After three times of freeze-degas-thaw, the reaction was carried out at 40 °C for 72 h. After that, the reaction mixture was precipitated with cold diethyl ether, then filtered and dried in vacuum. According to the $^1$H NMR, the polymer was determined as $PCL_{40}$-*b*-$PLL(Z)_{18}$. $PCL_{40}$-*b*-$PLL(Z)_{18}$ (1 g, 0.1 mmol), trifluoacetic acid (1 mL), hydrobomic acid in acetic acid (30% wt, 1 mL) and 5 mL trichloromethane were added into a round-bottom flask. After stirring for 24 h at r.t., the mixture was precipitated with cold diethyl ether, then filtered and dried in vacuum.

### 3.3 Synthesis of PCL-*b*-PLL-*g*-NBCF

PCL-*b*-PLL-*g*-NBCF was synthesized by chemical conjugation of NBCF to PCL-*b*-PLL. The reaction progress was shown in Fig. S2. $PCL_{40}$-*b*-$PLL_{18}$ (0.5 g, 0.05 mmol) and DIPEA were suspended in DMF and stirring at 0 °C for 30 min. NBCF (0.29 g, 1.35 mmol) was dissolved in DMF and added dropwise into the reaction solution. After stirring for 12 h at r.t., the crude products were purified by dialysis in the DMF and water (v/v = 1 : 1) for 1 day to remove the excessive NBCF and then dialysis in pure water for two days to remove DMF. According to the $^1$H NMR in Figure S9, the modified degree was about 98% and that means most of the PLL has been modified by the NBCF.

### 3.4 Synthesis of PCL-*b*-PEG-Cy5

PCL-*b*-PEG-Cy5 was synthesized according to the previous reports and the synthesis routes were described in Fig. S3. First, PCL-*b*-PEG-Boc was synthesized from Boc-PEG-OH and ε-caprolactone, followed by deprotection of Boc-PEG-OH to obtain PCL-*b*-PEG-$NH_2$. Then Cy5 NHS was conjugated to $PCL_{40}$-*b*-$PEG_{114}$-$NH_2$ by acylation of the amino group.

Briefly, Boc-$PEG_{114}$-OH (2 g, 0.4 mmol), *ε*-caprolactone (2 g, 17.6 mmol), 20 μL $Sn(Oct)_2$ and 10 mL toluene was added into the Schlenk tube. The detailed reaction progress was similar as the previous procedure of PCL-*b*-PEG. PCL-*b*-PEG-Boc (4.0 g, 0.29 mmol) was dissolved in 15 mL dichloromethane, and then trifluoroacetic acid (150 μL, 1.98 mmol) was added into the solution. After stirring for 24 h at r.t., the reaction mixture was precipitated in cold diethyl ether and dried under vacuum to afford PCL-*b*-PEG-$NH_2$. According to the $^1H$ NMR result, the polymer was determined as $PCL_{40}$-*b*-$PEG_{114}$-$NH_2$. Cy5 NHS ester (5.6 mg, 6.6 μmol), $PCL_{40}$-*b*-$PEG_{114}$-$NH_2$ (200 mg, 14.8 μmol) and trimethylamine (2 μL, 13.2 μmol) were dissolved in DMF. After stirring for 48 h at r.t., the solution was dialyzed in pure water for 2 days. $PCL_{40}$-*b*-$PEG_{114}$-Cy5 was obtained by lyophilisation.

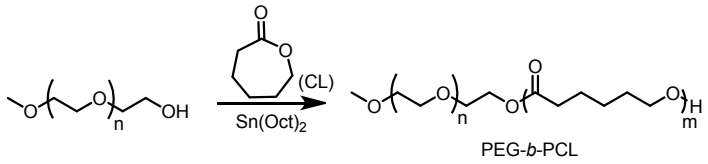


**Fig. S1.** Synthesis of $PCL_{40}$-*b*-$PEG_{114}$.

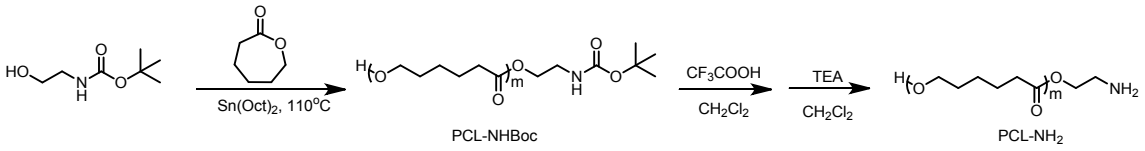


**Fig. S2.** Synthesis of $PCL_{40}$-$NH_2$

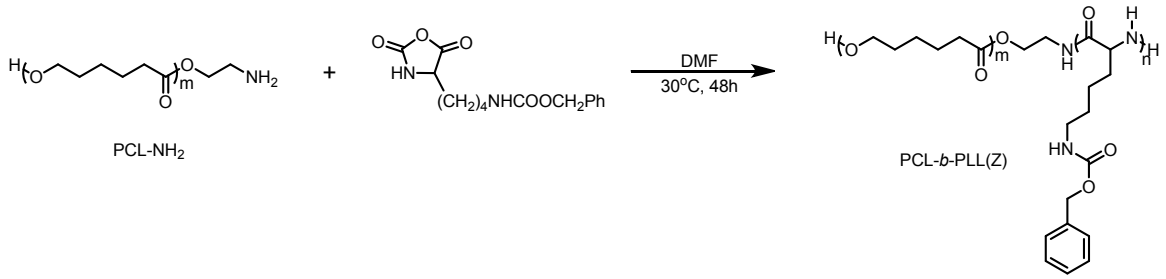


**Fig. S3.** Synthesis of $PCL_{40}$-*b*-$PLL(Z)_{18}$.

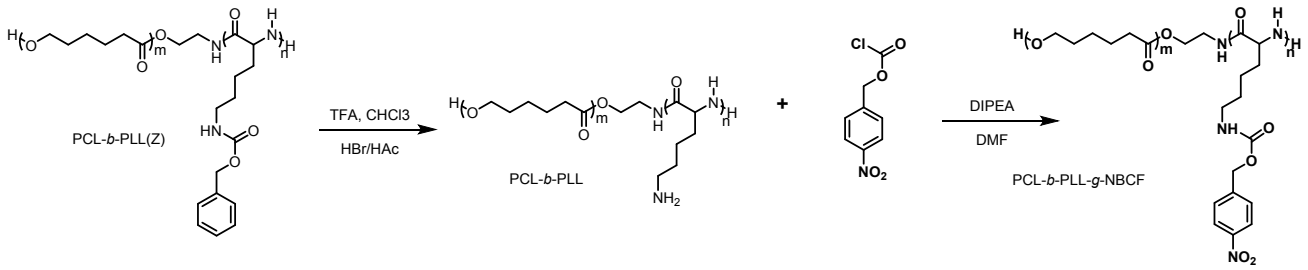


**Fig. S4.** Synthesis of PCL-*b*-PLL-*g*-NBCF.

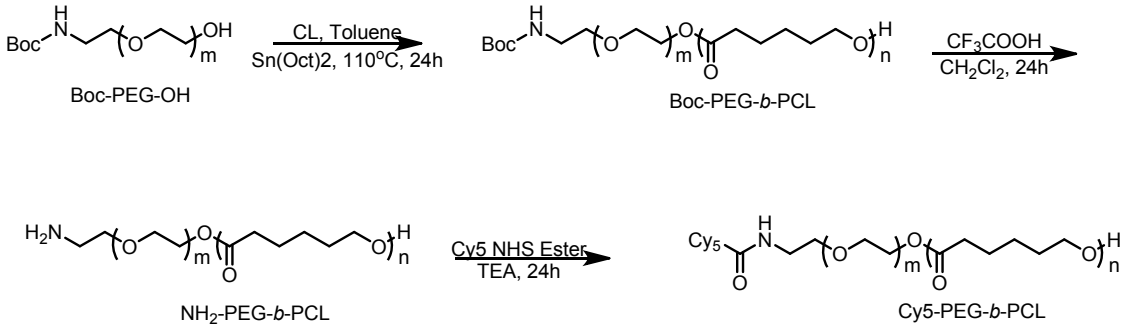


**Fig. S5.** Synthesis of $PCL_{40}$-*b*-$PEG_{114}$-Cy5.

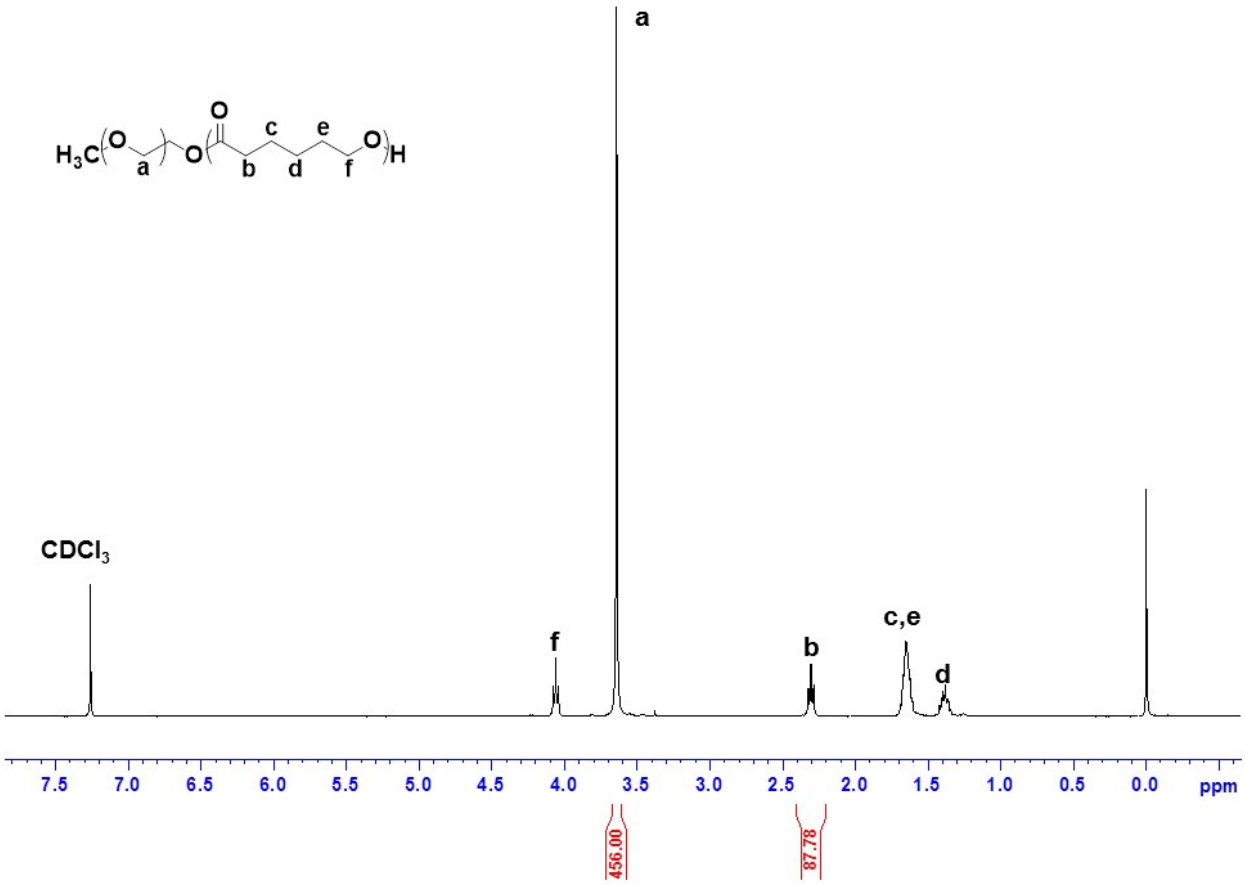


**Fig. S6.** $^1$H NMR spectra of $PCL_{40}$-*b*-$PEG_{114}$ in $CDCl_3$.

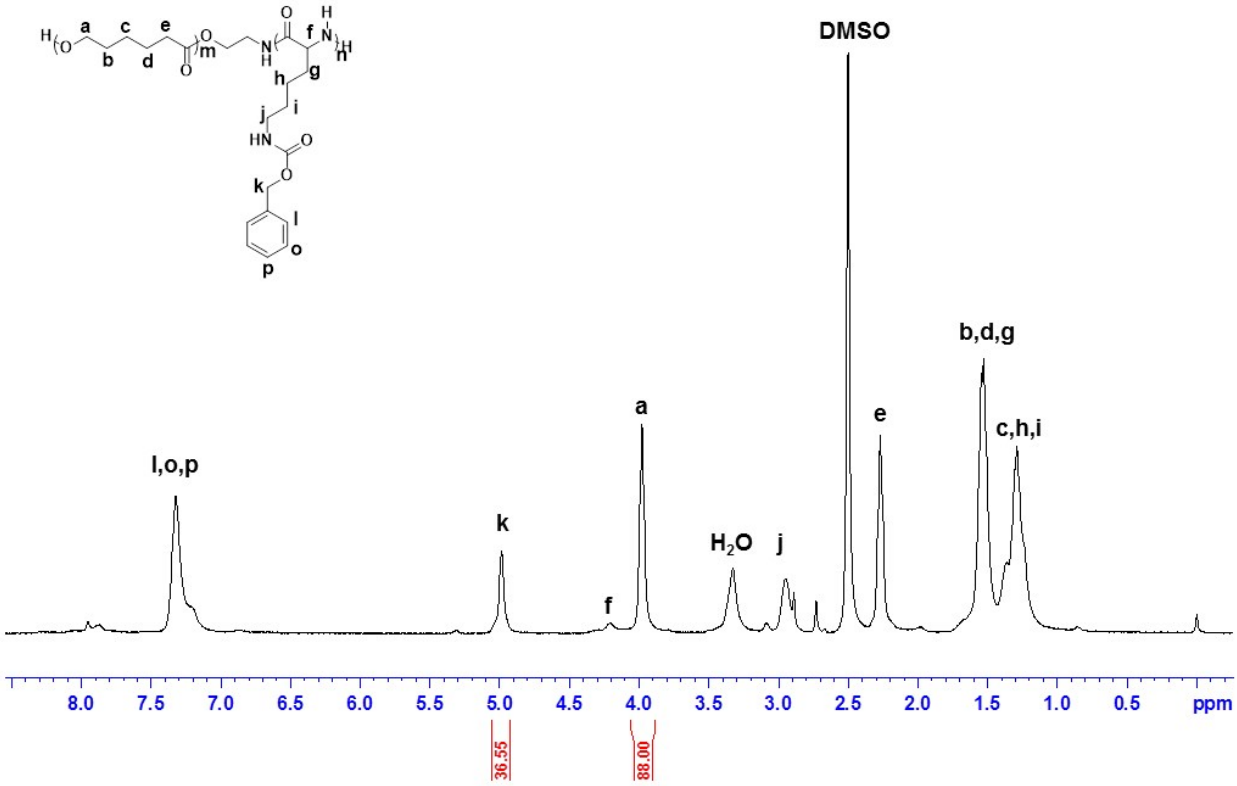


**Fig. S7.** $^{1}$H NMR spectra of $PCL_{40}$-*b*-$PLL(Z)_{18}$ in DMSO.

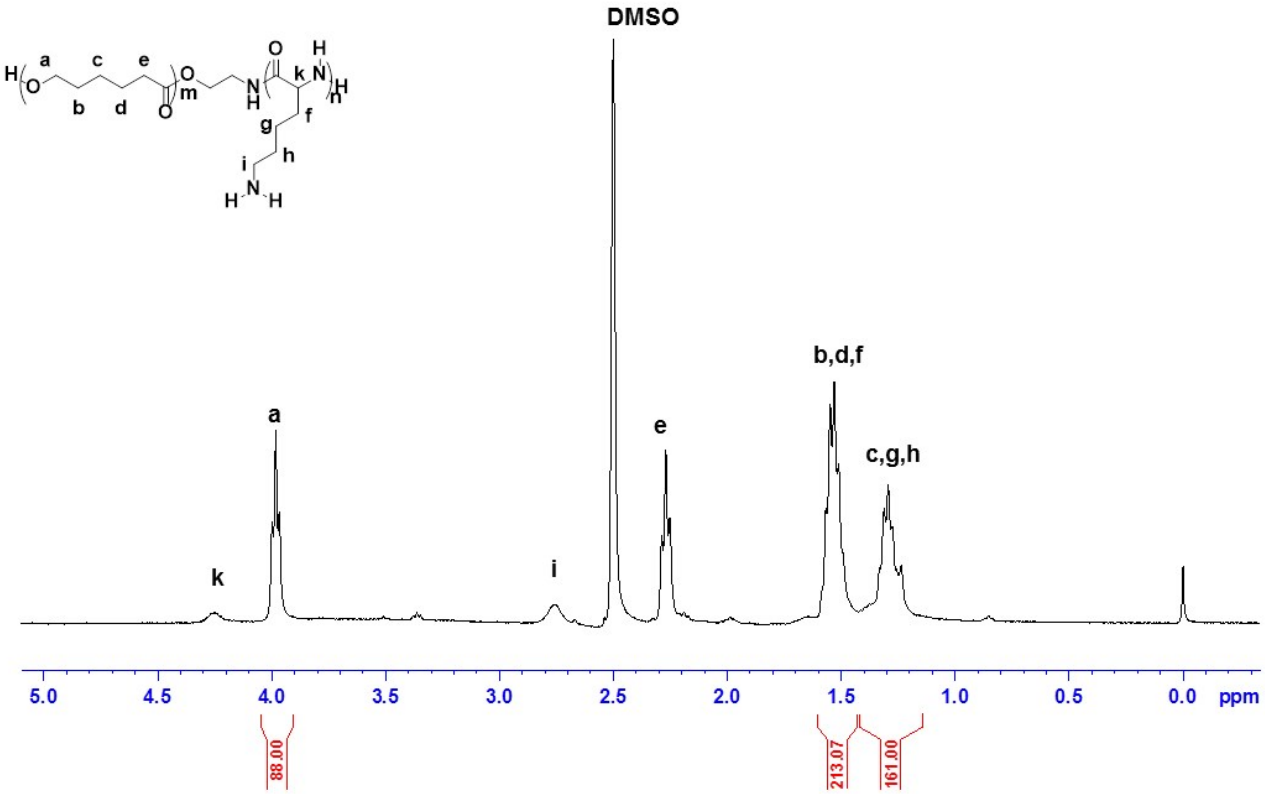


**Fig. S8.** $^{1}$H NMR spectra of $PCL_{40}$-*b*-$PLL_{18}$ in DMSO.

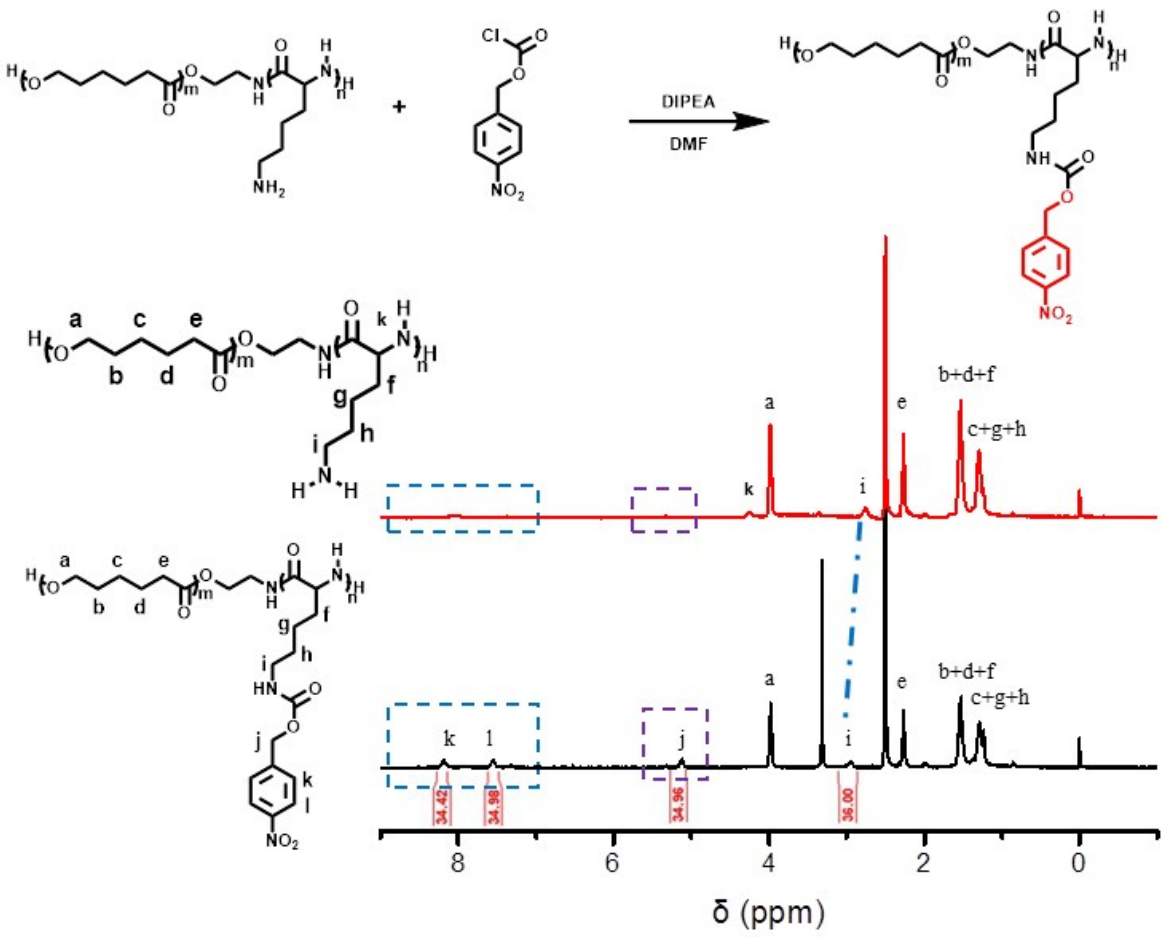


**Fig. S9.** $^{1}$H NMR spectra of $PCL_{40}$-*b*-$PLL_{18}$-*g*-NBCF in DMSO.

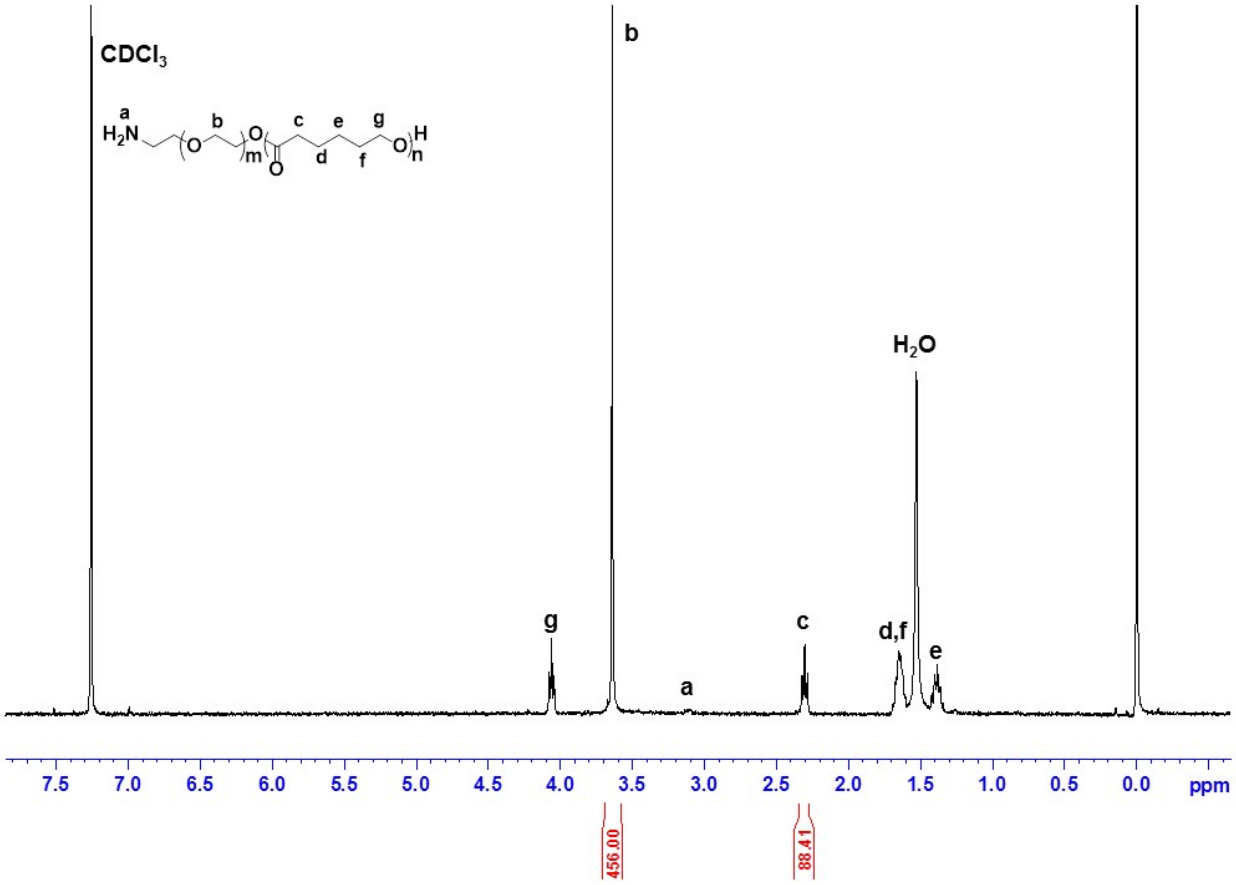


**Fig. S10.** $^{1}$H NMR spectra of $PCL_{40}$-*b*-$PEG_{114}$-$NH_2$ in $CDCl_3$

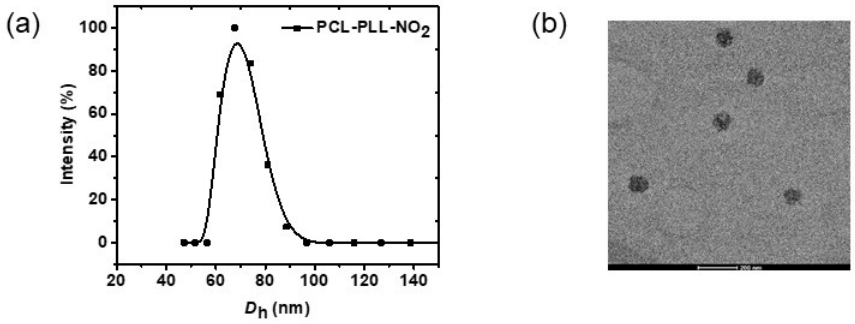


**Fig. S11.** (a) The hydrodynamic diameters of PM assembled by PCL-*b*-PLL-*g*-NBCF. The scale bar is 200 nm. (b) TEM images of PM.

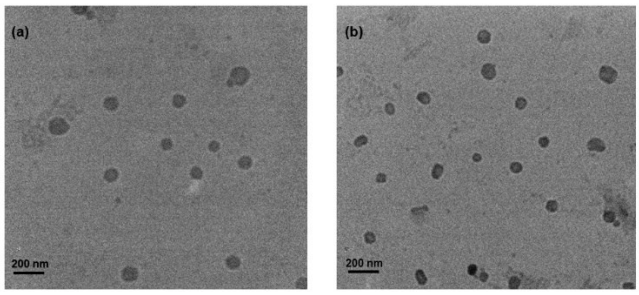


**Fig. S12.** TEM images of (a) RM and (b) NRM.

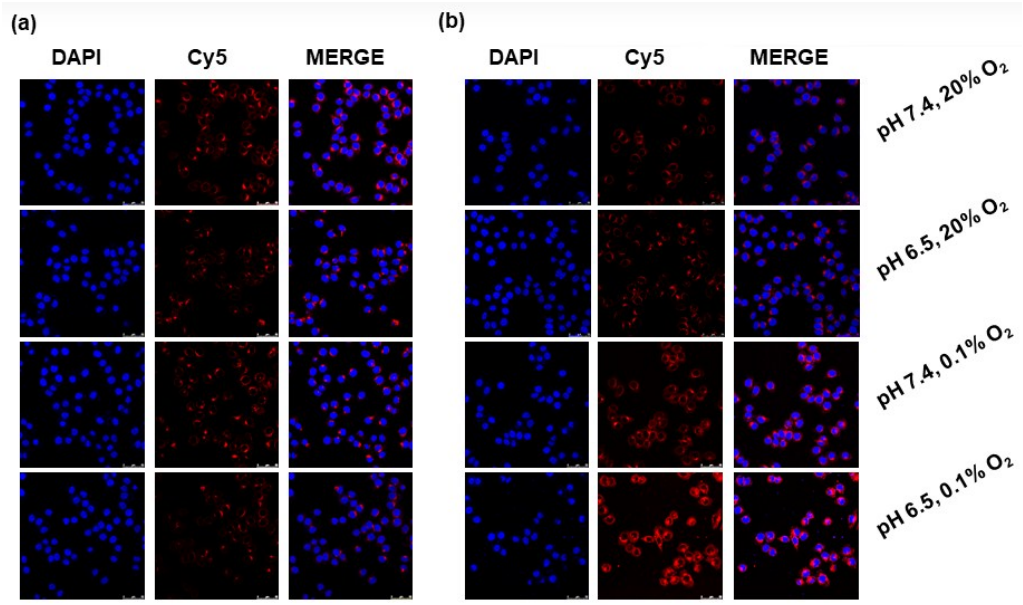


**Fig. S13.** (a) Confocal microscopy image of MDA-MB-231 cells after incubation with Cy5-NRM and (b) Cy5-RM at pH 7.4 and pH 6.5. Samples were incubated under normoxic and hypoxic conditions for 3 h.

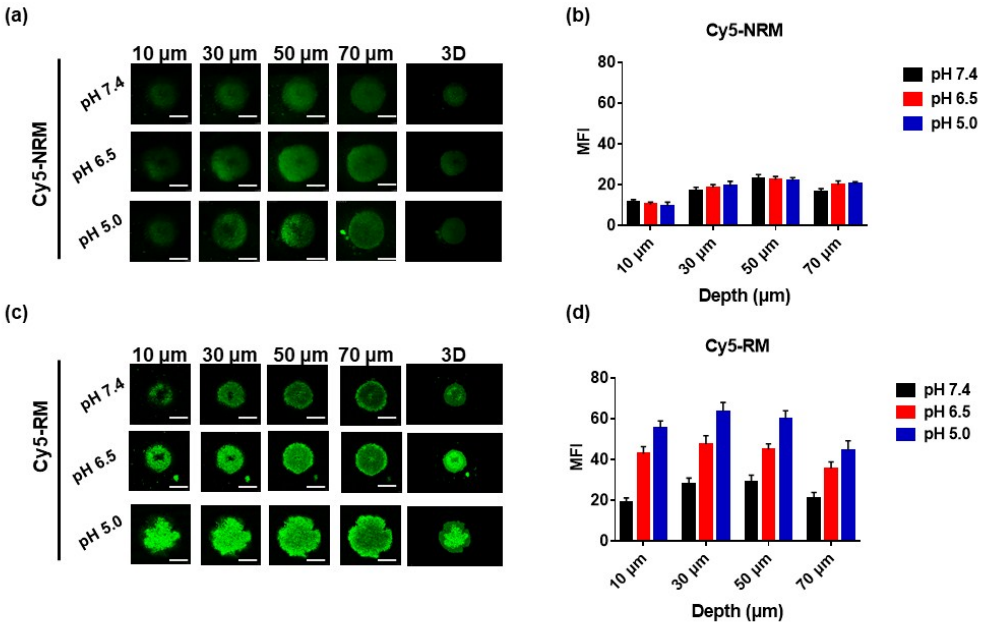


**Fig. S14. Penetration behavior of Cy5-NRM and Cy5-RM under different pH gradients.** (a) and (c) Confocal microscopy images of MCSs after incubated under different pH (0.1% $O_2$). The scale bar is 200 μm. (b) and (d) The MFI of MCSs quantitatively analysed by Image J.

| polymers | Transmittance (%) |
|---|---|
| $PCL_{5k}$ | 4.1±0.1 |
| $PCL_{5K}$-*b*-$PLL_{5K}$-*g*-NBCF | 15.2±0.2 |

**Table. S1.** The transmittance of $PCL_{5k}$ and $PCL_{5K}$-*b*-$PLL_{5K}$-*g*-NBCF in acid water.

| DOX-loaded micelles | DLC(wt%) | DLE(%) |
|---|---|---|
| NRM-DOX | 12.4 | 52.4 |
| RM-DOX | 11.5 | 47.5 |

**Table S2.** DLC (%) and drug loading efficiency (DLE) (%) of micelles.

1. H. L. Ou, T. J. Cheng, Y. M. Zhang, J. J. Liu, Y. X. Ding, J. R. Zhen, W. Z. Shen, Y. J. Xu, W. Z. Yang, P. Niu, J. F. Liu, Y. L. An, Y. Liu and L. Q. Shi, Acta biomater., 2018, **65**, 339-348.
2. F. Huang, L. L. Shen, J. Z. Wang, A. T. Qu, H. R. Yang, Z. K. Zhang, Y. L. An and L. Q. Shi, *ACS Appl. Mater. Interfaces*, 2016, **8**, 3669-3678.