

Supporting Information

Rapid Release from Near-Infrared Polymer Loaded Liposomes for Photothermal and Chemo- Combined Therapy

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Experimental Section

1. Materials

2-thiophenenitrile (99%), 2-bromothiophene (97%), tetramethylethylenediamine (~99%), trimethyltin chloride (>98.0%), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), anhydrous potassium carbonate ($\geq 99\%$), tri(*o*-tolyl)phosphine (97%), tris(dibenzylideneacetone)dipalladium(0) and Brij®S20 were purchased from Sigma-Aldrich. diisopropyl succinate (> 99.0%), tert-amyl alcohol (> 98.0%), potassiumtert-butoxide (> 97.0%), 7-(bromomethyl)pentadecane (> 95.0%) and *N*-bromosuccinimide (> 98.0%) were purchased from TCI. All of the compounds were used as received. The solvents for chemical syntheses were purchased from commercial sources and purified by distillation. Chemical reactions were in progress under an argon atmosphere. All of the syntheses and characterizations were given in the supporting information.

2.Characterizations

¹H NMR spectra were measured at room temperature by a Unity-400 MHz NMR

spectrometer (Bruker). Diameters were performed with a Brookhaven 90Plus size analyzer. Transmission electron microscopy (TEM) images were recorded on a JEOL JEM-1011 electron microscope. Confocal laser scanning microscopy (CLSM) images were visualized with a Zeiss 710 confocal laser scanning microscopy image system. UV–visible absorption spectra were recorded via a Varian Cary 300 UV–visible spectrophotometer in 1 cm path-length cuvette.

3. Calculation of Photothermal Conversion Efficiency

Following Roper’s report, the total energy balance for the system can be expressed by Eq. 1:

$$\sum m_i C_{p,j} dT/dt = Q_{NC} + Q_{dis} - Q_{surr} \quad (1)$$

where m and C_p are the mass and heat capacity of water, respectively. T is the solution temperature, Q_{NC} is the energy inputted by nanoparticles, Q_{Dis} is the baseline energy inputted by the sample cell, and Q_{Surr} is heat conduction away from the system surface by air.

The laser-induced source term, Q_{NC} , represents heat dissipated by electron-phonon relaxation of the plasmons on the nanoparticles surface under the irradiation of 808 nm laser:

$$Q_{NC} = I (1 - 10^{-A_{808}}) \eta \quad (2)$$

where I is incident laser power, η is the conversion efficiency from incident laser energy to thermal energy, and A_{808} is the absorbance of the nanoparticles at wavelength of 808 nm (Fig. 2C). In addition, source term, Q_{Dis} , expresses heat dissipated from light absorbed by the quartz sample cell itself, and it was measured independently to be 1.1 W using a quartz cuvette cell containing pure water without nanoparticles. Furthermore, Q_{Surr} is linear with temperature for the outgoing thermal energy, as given by Eq. 3:

$$Q_{Surr} = hS(T - T_{surr}) \quad (3)$$

where h is heat transfer coefficient, S is the surface area of the container, and T_{Surr} is ambient temperature of the surrounding. Once the laser power is defined, the heat input ($Q_{NC} + Q_{Dis}$) will be finite. Since the heat output (Q_{Surr}) is increased along with the increase of the temperature according to the Eq. 3, the system temperature will rise to a maximum when the heat input is equal to heat output:

$$Q_{NC} + Q_{Dis} = Q_{Surr-Max} = hS(T_{max} - T_{surr}) \quad (4)$$

where the $Q_{Surr-Max}$ is heat conduction away from the system surface by air when the sample cell reaches the equilibrium temperature, and T_{max} is the equilibrium temperature. The 808 nm laser heat conversion efficiency (η) can be determined by substituting Eq.2 for Q_{NC} into Eq. 4 and rearranging to get

$$\eta = \frac{hS(T_{max} - T_{surr}) - Q_{Dis}}{I(1 - 10^{-A_{808}})} \quad (5)$$

where Q_{Dis} was measured independently to be 1.1 W, the $(T_{max} - T_{Surr})$ were 23 °C according to Fig. 2C, I is 3.0 W/cm², A_{808} is the absorbance (0.4) of nanoparticles at 808 nm. Thus, only the h_s remains unknown for calculating η . In order to get the h_s , a dimension less driving force temperature, θ is introduced using the maximum system temperature, T_{max}

$$\theta = \frac{(T - T_{surr})}{(T_{max} - T_{surr})} \quad (6)$$

and a sample system time constant τ_s

$$\tau_s = \frac{\sum_i m_i C_{pj}}{hS} \quad (7)$$

which is substituted into Eq. 1 and rearranged to yield

$$\frac{d\theta}{dt} = \frac{1}{\tau_s} \left[\frac{Q_{NC} + Q_{Dis}}{hS (T_{max} - T_{surr})} - \theta \right] \quad (8)$$

At the cooling stage of the aqueous dispersion of the nanoparticles, the light source was shut off, the $Q_{NC} + Q_{Dis} = 0$, reducing the Eq.9

$$dt = -\tau_s \frac{d\theta}{\theta} \quad (9)$$

and integrating, giving the expression

$$t = -\tau_s \text{Ln}\theta \quad (10)$$

Therefore, time constant for heat transfer from the system is determined to be $\tau_s = 263.0$ s by applying the linear time data from the cooling period vs negative natural

logarithm of driving force temperature (Fig.2D). In addition, the m is 5.0 g and the C is 4.2 J/g. Thus, according to Eq. 7, the h_s is deduced to be 0.0798 W/°C substituting them into Eq. 5, the 808 nm laser heat conversion efficiency (η) of nanoparticles can be calculated to be 38.9%.

4. Liposome preparation

Coencapsulated liposomes were prepared using a method described previously with some minor modifications. Three types of liposomal formulations were prepared: 15.0 mg of lipids mixed with surfactant (DPPC/Brij®S20 = 96/4, molar ratio) were dissolved in isopropanol (IPA) and dried at 65 °C under gentle stream of nitrogen gas, and the resulting thin lipid film was placed under high vacuum for at least 2 h to remove residual organic solvent. The thin lipid film was hydrated with 300 mM citric acid (pH = 2) to obtain 20 mM liposomes spension. After sonication and membrane extrusion (at 65°C, 200 nm membrane) to control the size, the liposomes were cooled to room temperature. The exterior buffer of the liposome suspension was replaced by HBS (25 mM HEPES Buffered Biotechnology, Rockford, IL) for 3 h against three exchanges of 500×volumes of HBS at room temperature. The liposome suspension and DOX were mixed at 1:10 (w/w, drug/lipid), and the mixture was incubated at 37°C for 90 min. Then, PDPPT was dissolved in 2.0 mL THF and added in liposome solution (1:10 (w/w, PDPPT/lipid)). THF was removed by dialysis against HBS for 24 h. The dialysis medium was refreshed five times and whole procedure was performed in the dark. Then, the solution was filtered and lyophilized. To determine the loading content (LC) of DOX and PDPPT, the coencapsulated liposomes were dissolved in DMSO analyzed by UV absorption measurement using a

standard curve method. LC of coencapsulated liposomes were calculated according to eqn (11):

$$\text{LC (wt \%)} = \text{amount of drug in coencapsulated liposomes} / \text{amount of coencapsulated liposomes} \times 100 \quad (11)$$

Triggered release of DOX from coencapsulated liposomes at physiological body temperature (37°C) was measured. 1.0 mg mL⁻¹ of coencapsulated liposomes was added to a glass tube and the sample was irradiated with a NIR laser of 808 nm at different powers and irradiation times. The release of DOX from coencapsulated liposomes was measured by fluorescence spectro-photometry ($\lambda_{\text{ex}} = 480$ nm). The percentage of coencapsulated liposomes released DOX was calculated according to the formula:

$$\text{Drug release (\%)} = (F_t - F_0) / (F_{\text{max}} - F_0) \times 100 \quad (12)$$

where F_t is the DOX fluorescence intensity of the liposome, F_0 is the initial background DOX fluorescence intensity of the liposome, and F_{max} is the fluorescence intensity of DOX in liposomes after the dissolution of DOX-loaded liposomes in organic solvent mixture (chloroform and methanol = 4:1, v/v). Drug release test was performed using three independent samples of each liposomal formulation.

5. Cell Proliferation and Apoptosis Analyse

HepG2 cells were seeded in 6-well culture plates and divided into four groups treated with PBS, PBS+NIR, PDPPT-Lip and PDPPT-Lip+NIR irradiated, respectively. After NIR light irradiation of 3.0 W cm⁻¹ for 5 min, all HepG2 cells were incubated for further 4 h at 37°C in the dark. Cells incubated with PBS without NIR

light irradiation served as comparison. To detect cell apoptosis, HepG2 cells were collected. Then the cells were stained by using an annexin V-FITC and PI staining kit from manufacturer's instructions (BD, USA). The induction of apoptosis in HepG2 cells were examined by a FACS Calibur flow cytometer (BD Biosciences).

6. Cytotoxicity Assay

The cytotoxicities of coencapsulated liposomes against HepG2 were evaluated *in vitro* by a MTT assay. The cells were seeded in 96-well plates at 1×10^4 cells per well in 200 μL of complete DMEM and incubated at 37°C in 5.0 % CO_2 atmosphere for 24 h. The culture medium was then removed and coencapsulated liposomes in complete DMEM at different concentrations were added. For PTT experiment, the cells were treated with an 808 nm laser at a power density of 3.0 W cm^{-2} for 5 minutes. The cells were subjected to MTT assay after being incubated for additional 48 h. The absorbance of the solution was measured on a Bio-Rad 680 microplate reader at 490 nm. Cell viability (%) was calculated based on eq: (13).

$$\text{Cell viability (\%)} = A_{\text{sample}} / A_{\text{control}} \times 100 \quad (13)$$

where A_{sample} and A_{control} represent the absorbances of sample and control wells, respectively.

7. Confocal Laser Scanning Microscopic Observation

HepG2 cells were seeded in 6-well plates at a density of 2×10^5 cells per well in 2.0 mL of complete Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, supplemented with 50 IU mL^{-1} penicillin and 50 IU mL^{-1} streptomycin, and cultured for 24 h. After the culture medium was removed, complete

DMEM containing coencapsulated liposomes at a final DOX concentration of 5.0 mg L⁻¹ was added. For PTT experiment, the cells were treated with an 808 nm laser at a power density of 3.0 W/cm² for 5 minutes. The cells were incubated at 37°C for additional 2 h. Then, the culture medium was removed and cells were washed with PBS three times. The cells were fixed with 4% para-formaldehyde for 30 min at room temperature, and the cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, blue) for 20 min. Confocal laser scanning microscope (CLSM) images of cells were obtained through confocal microscope (Olympus Fluo View 1000).

8. Flow Cytometric Analyses

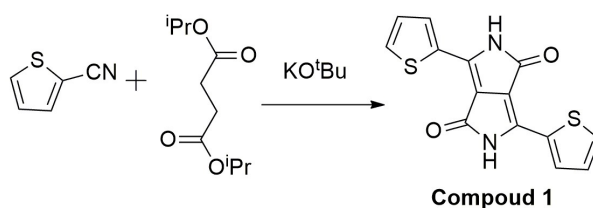
HepG2 cells were seeded in 6-well plates at 2×10^5 cells per well in 2.0 mL of complete DMEM, and cultured for 24 h. The culture medium was then removed, and complete DMEM containing coencapsulated liposomes at a final DOX concentration of 5.0 mg L⁻¹ was added. After the cells were incubated at 37°C for additional 2 h, the culture medium was removed and the cells were treated with trypsin. Then, 1.0 mL of PBS was added to each culture well, and the solutions were centrifuged for 4 min at 3000 rpm. After removing the supernatant, the cells were resuspended with 0.3 mL of PBS. Data for 1×10^4 gated events were collected, and analysis was performed by FACS Calibur flow cytometer (BD Biosciences).

9. Live/Dead Assay

To further visualize the cell cytotoxicity of chemothermal, photothermal and chemophotothermal treatments, HepG2 cells were seeded into 24-well plates (5×10^4 cells well⁻¹) and incubated overnight. The cells were then replaced with fresh media

containing different liposomes. After 24 h of incubation, cells treated with liposomes were exposed to 808 nm laser of 3.0 W cm^{-2} . The groups treated with PBS and laser irradiation were served as a control. After another 4 h, cells were washed with PBS and stained with calcein-AM for visualization of live cells and with PI for visualization of dead/late apoptotic cells. The cells were examined by an inverted fluorescence microscope system (Olympus, Japan).

10. Synthesis



Scheme S1. Synthesis of 3,6-Bis(thiophen-2-yl)-2H,5H-pyrrolo[3,4-c]pyrrole-1,4-dione.

To a three-neck round-bottom flask, tert-amyl alcohol (80 mL) and potassium tert-butoxide (15.5 g, 0.1 mM) were added under an Ar atmosphere. The flask was equipped with a mechanical stirrer and a reflux condenser. The mixture was heated at 110°C for 1 h under argon atmosphere. 2-thiophenenitrile (11.0 g, 0.1 M) was added and stirred at 110°C for 30 min. A mixture of diisopropyl succinate (8.1 g, 40 mM) in tert-amyl alcohol (12 mL) was added dropwise over a period of 3 h with rapid stirring. The mixture was then stirred at 110°C for a further 2 h, and cooled to room temperature. The mixture was poured into the mixture of 120 g of ice, hydrochloric

acid (35.0% aq.) (32 mL) and methanol (160 mL) and the mixture was stirred for 45 min. The mixture was filtered and the solid was washed with methanol (30 mL). The solid was dried under vacuum at 60°C overnight to give the product as a dark red solid (14.0 g, yield 85.0%). ¹H NMR (400 MHz, DMSO-d₆, 25°C) δ(ppm): 11.25 (s, 2H), 8.25 (d, 2H), 7.96 (d, 2H), 7.30 (t, 2H).

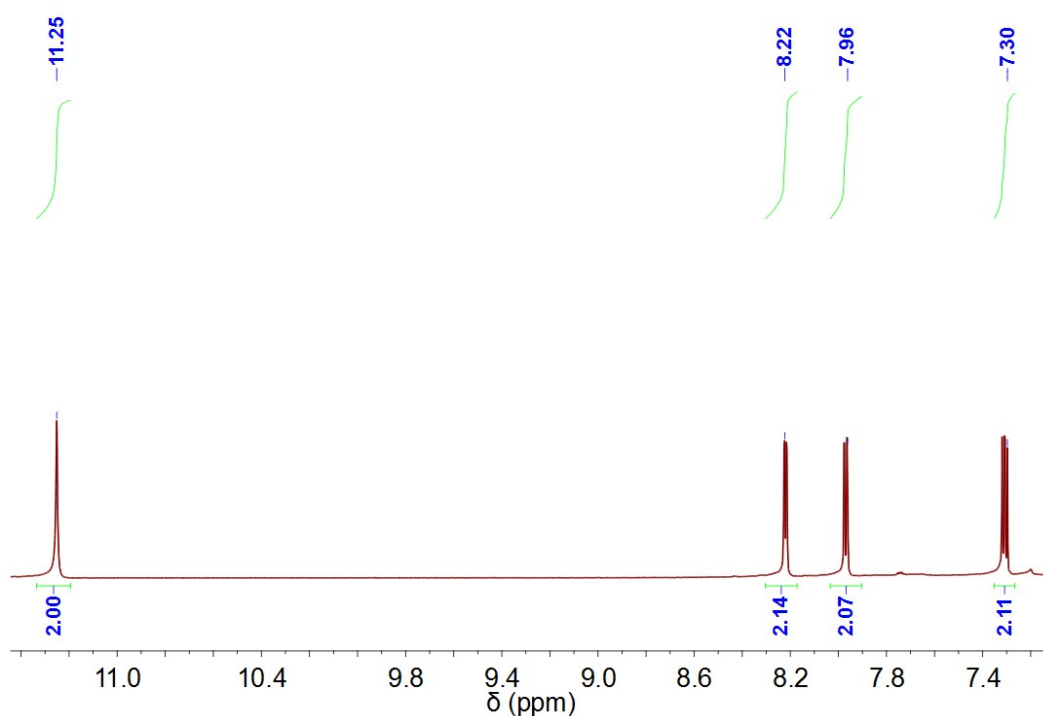
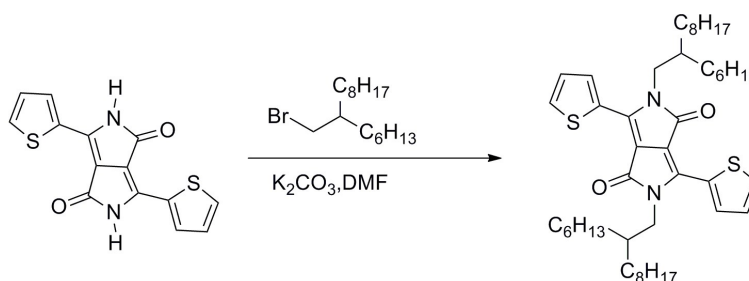


Figure S1. ¹H NMR spectrum of 3,6-Bis(thiophen-2-yl)-2H,5H-pyrrolo[3,4-c]pyrrole-1,4-dione.



Scheme S2. Synthesis of 2,5-Dihexadecyl-3,6-bis(thiophen-2-yl)pyrrolo[3,4-

c]pyrrole-1,4-dione

3,6-Bis(thiophen-2-yl)-2H,5H-pyrrolo[3,4-c]pyrrole-1,4-dione (1.5 g, 5.0 mM) and sodium hydride (0.5 g, 20.0 mM) were added to anhydrous N,N-dimethylformamide (DMF) (30 mL) under argon and stirred at 130°C for 1 h. Then 7-(Bromomethyl)pentadecane (5.4 g, 25.0 mM) was added and the reaction mixture stirred at 130°C overnight. The reaction mixture was cooled to room temperature and added water to remove residual sodium hydride. The solvent was removed under reduced pressure and the resulting tacky solid was purified by column chromatography using petroleum ether: dichloromethane (1:3) as the eluent. The product was dried under vacuum to give a dark red solid (0.3 g, yield 13.0%). ¹H NMR (400 MHz, CDCl₃, 25°C) δ (ppm): 8.86 (d, 2H), 7.61 (d, 2H), 7.28 (t, 2H), 4.03(d, 4H), 1.90 (br, 2H), 1.21 (m, 48H), 0.84 (t, 12H).

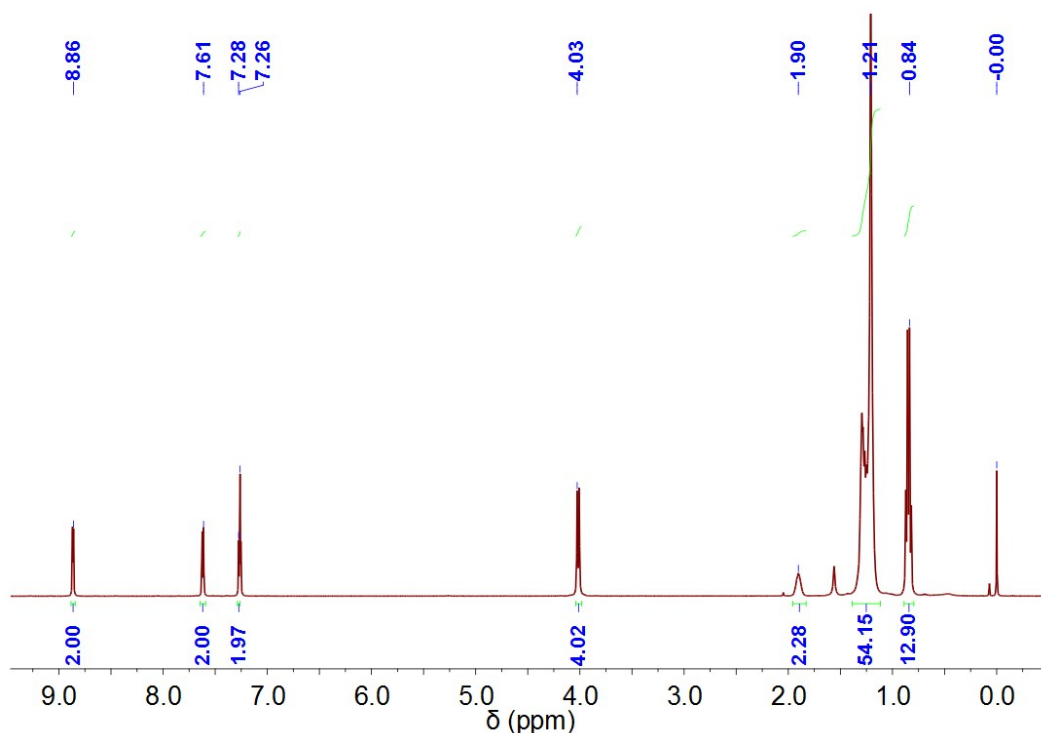
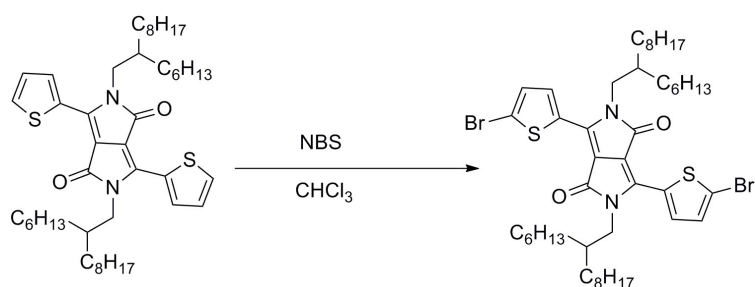


Figure S2. ^1H NMR spectrum of 2,5-dihexadecyl-3,6-bis(thiophen-2-yl)pyrrolo[3,4-*c*]pyrrole-1,4-dione in CDCl_3 .



Scheme S3. Synthesis of 3,6-bis(5-bromothiophen-2-yl)-2,5-dihexadecylpyrrolo [3,4-*c*]pyrrole-1,4-dione.

2,5-Dihexadecyl-3,6-bis(thiophen-2-yl)pyrrolo[3,4-*c*]pyrrole-1,4-dione (0.3 g, 0.5 mM) and chloroform (10 mL) were added in a flask. The flask is pre-heated at 50 °C for 30 min. *N*-bromosuccinimide (0.2 g, 1.3 mM) was added to the flask. The reaction mixture was stirred at 50 °C for about 2 h. Then the chloroform was evaporated, and the resulting dark red solid was purified by column chromatography using n-hexane: dichloromethane (1:2) as the eluent to give the product as a red solid (0.2 g, yield 79.9%). ^1H NMR (400 MHz, CDCl_3 , 25 °C) δ (ppm): 8.62 (d, 2 H), 7.22 (d, 2 H), 3.93 (d, 4 H), 1.88 (br, 2 H), 1.22(m, 48 H), 0.86 (m, 12 H).

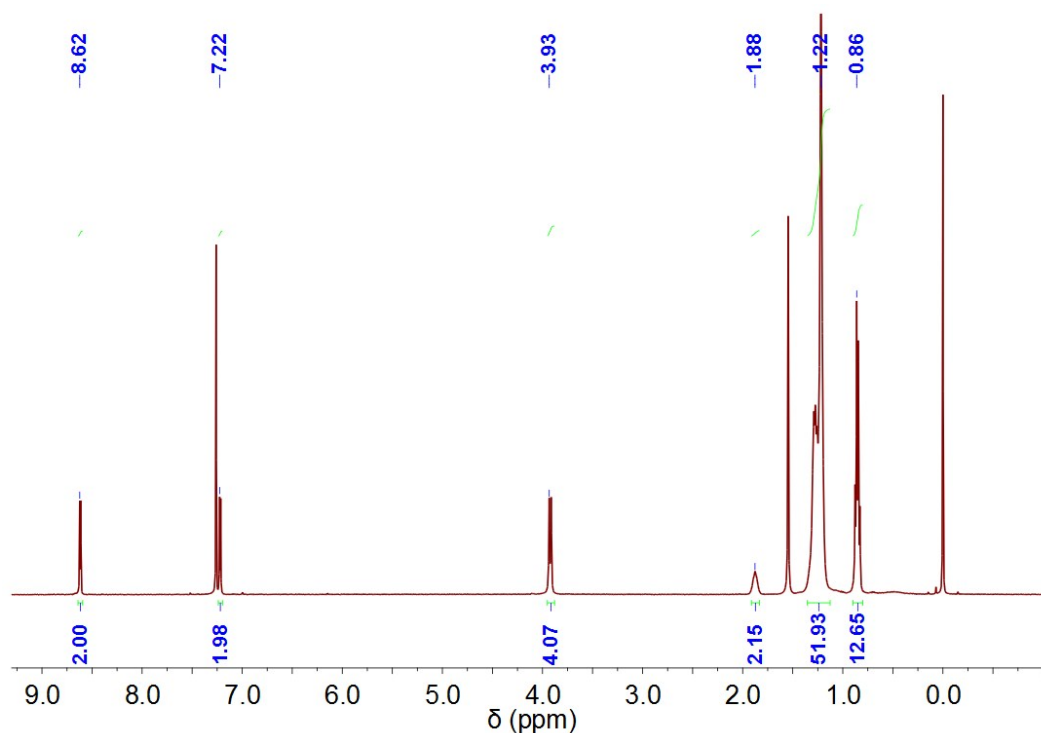
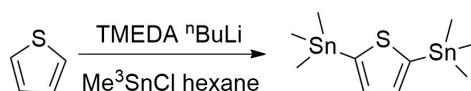


Figure S3. ¹H NMR spectrum of 3,6-bis(5-bromothiophen-2-yl)-2,5-dihexadecyl pyrrolo[3,4-c]pyrrole-1,4-dione.



Scheme S4. 2,5-Bis(trimethylstannyl) thiophene

To a 250 mL three-neck round-bottom flask, 2.0 mL (10 mM) of thiophene, 8.8 mL (20.5 mmol) of tetramethylethylenediamine (TMEDA) and 25 mL hexane were added under argon at 0°C. 8.2 mL (2.5 M, 20.5 mM) of n-butyl lithium was dropwise added to the flask. The reaction mixture was heated at reflux for 30 min, then cooled to 0°C followed to add dropwise 20.5 mL of trimethyltin chloride in hexane (1.0 M). Then

the mixture reached to room temperature and stirred overnight. The mixture was quenched with water, extracted with hexane and washed with water several times. The organic phase was dried with MgSO_4 and the solvent was removed under reduced pressure to give a pale-white solid. Recrystallization from ethanol gave the product as a white crystal (3.3 g, yield 81.1%). ^1H NMR (400 MHz, CDCl_3 , 25 °C) δ (ppm): 7.37 (s, 2 H), 0.36 (t, 18 H).

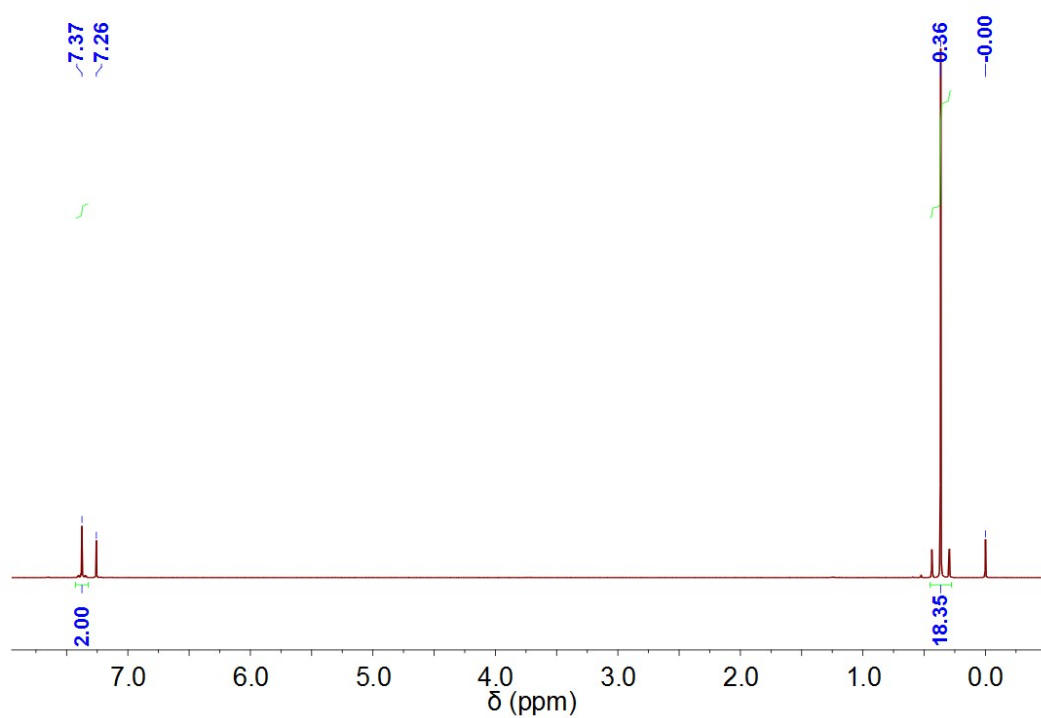
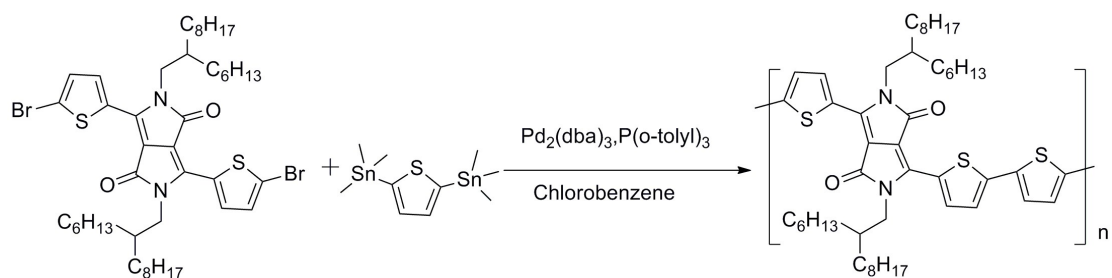


Figure S4. ^1H NMR spectrum of 2,5-Bis(trimethylstannyl) thiophene



Scheme S5. Synthesis of PDPPT.

A 50 mL Schlenk tube was heated under reduced pressure and then allowed to cool to room temperature at nitrogen. 2,5-bis(trimethylstannyl)thiophene (123 mg, 0.3 mM) 2,5-bis(4-bromobutyl)-3,6-bis(5-bromothiophen-2-yl)pyrrolo[3,4-c]pyrrole-1,4 (2H, 5H)-dione (218 mg, 0.3 mM) and dry chlorobenzene (15 mL) were added to this tube. The solution was degassed with argon for 30 min, followed by addition of $\text{Pd}_2(\text{dba})_3$ (15 mg) and $\text{P}(\text{o-tol})_3$ (60 mg) and then degassed and charged with argon several times. The reaction mixture was stirred vigorously at 120°C for 72 h under argon. 2-Bromothiophene (30 mg, 0.2 mM) was then added and the reaction was continued for another 12 h. After cooling down to 80°C , aqueous solution of sodium diethyldithiocarbamate trihydrate was added to remove the residual palladium catalyst. After 12 h, the mixture was filtered and then extracted on a Soxhlet's extractor with acetone, hexane and chloroform successively. The final product was the residual palladium. The polymer was dried under reduced pressure at room temperature for at least 24 h to obtain a black solid, 204 mg, 84.9% yield. $(\text{C}_{26}\text{H}_{24}\text{Br}_2\text{N}_2\text{O}_2\text{S}_3)_n$. ^1H NMR (400 MHz, CDCl_3 , 25°C)

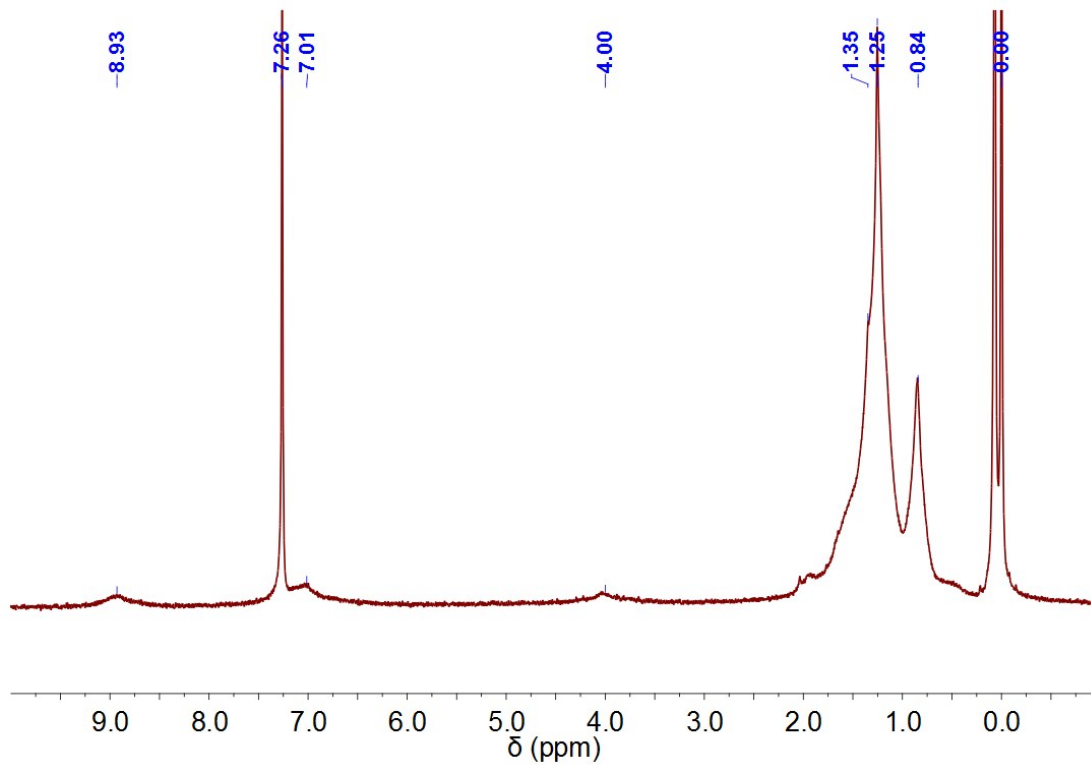


Figure S5. ¹H NMR spectrum of PDPPT.

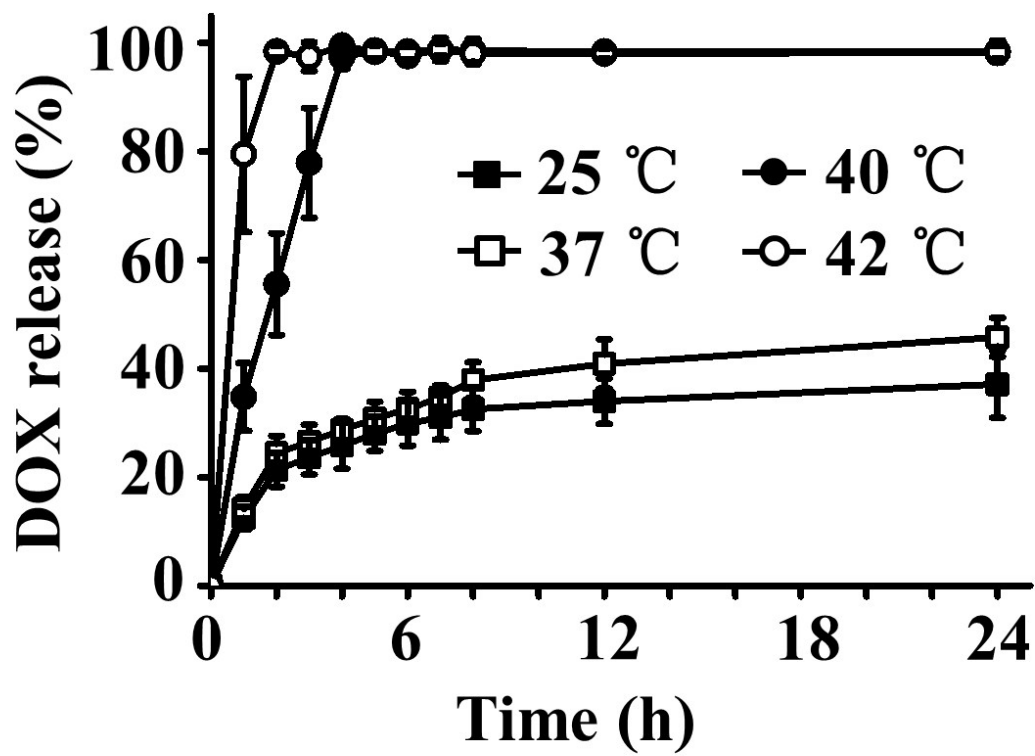


Figure S6. Time-dependent release of DOX from DOX/PDPPT-Lip at different temperature. The data are represented as a mean \pm S.D. (n = 3).