Electronic Supplementary Material (ESI) for Chemical Communications.
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Supplementary information

Hexagonal polypyrrole nanosheets from interface driven heterogeneous hybridization and self-assembly for photothermal cancer treatment

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A: Experimental Section

1. Chemicals and Materials

Unless otherwise noted, all reagent-grade chemicals were used as received, and distilled water was used for the preparation of all aqueous solutions. Tetrahydrofuran (THF), calcein, phosphate buffered saline (PBS, pH 7.4) and dopamine hydrochloride (DA, 98%) were purchased from Aladdin Chemical Co., Ltd (Shanghai, China) and used as received. Pluronic F127 ($EO_{106}PO_{70}EO_{106}$, Mw = 12600) was purchased from (Shanghai, China). Pyrrole (Py) was purchased from MACKLIN (Shanghai, China). Iron (III) chloride anhydrous ($FeCl_3$) was purchased from Alfa Aesar. 2-phenylethynesulfonamide (PES) was purchased from TCI. The human DOX resistant breast cancer MCF-7/ADR cell line was purchased from Bogoo Biological Technology Co., Ltd (Shanghai). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Chemical (Shanghai). Hoechst 33258 was purchased from Yeasen Biological Technology Co., Ltd (Shanghai).

2. Synthesis of FeOOH-polypyrrole nanosheets

The FeOOH-polypyrrole nanosheets (NS) was synthesized by a simple one-pot liquid synthesis strategy. In a typical synthesis, 75 mg of F127 was dissolved into 900 μL THF together with 65 μL of Py. After ultrasonication for three minutes, 3 mg of dopamine hydrochloride (DA) dissolved in deionized water (9 mL) was injected into the above mixture

solution. The solution was further sonicated for ten minutes and transferred to an open conical flask (250 mL in size). Finally, 1 mL of $FeCl_3$ (37 mg) aqueous solution was added and the reaction solution was stirred at room temperature for 24 h. The molar ratio of the reactants is 1 Py: 0.017 DA: 0.245 $FeCl_3$: 11.76 THF: 595.7 H_2O: 0.0064 F127. The final product of NS was obtained by dialysis against water for three days.

3. Synthesis of polypyrrole nanomaterials with altered recipes

Different molar ratios of DA/Py ranging from 0 to 0.045 were employed to investigate the influence of DA on the formation of the nanosheets. To explore the other parameters controlling the NS formation, altered recipes with: more $FeCl_3$ (46.25 mg), less THF (450 μL), lower temperatures (4 °C) and higher temperatures (40, 50 and 60 °C) were separately explored while keeping the other parameters unchanged.

4. Synthesis of polypyrrole nanoparticles

The polypyrrole nanoparticles (PPy) was also synthesized in the absence of dopamine, in order to compare with NS for demonstrating the advances in the structure and properties of the NS product. Typically, 75 mg of F127 was dissolved into 900 μL THF together with 65 μL of Py. After ultrasonication for three minutes, 9 mL deionized water was injected into the above mixture solution. The solution was further sonicated for ten minutes and transferred to an open conical flask (250 mL in size). Finally, 1 mL of $FeCl_3$ (37 mg) aqueous solution was added

and the reaction solution was stirred at room temperature for 24 h. The molar ratio of the reactants is 1 Py: 0.245 $FeCl_3$: 11.76 THF: 595.7 H_2O: 0.0064 F127.

5. Characterization of NS

Transmission electron microscopy (TEM) images were obtained using a JEM 2010 (JEOL, Japan) instrument with an acceleration voltage of 200 kV in order to investigate the size, morphology and integrity of the nanoparticles. Samples were dried on holey carbon-coated Cu grids.

Atomic force microscope (AFM) measurement was performed on an MFP-3D-BIO system (Asylum Research, US).

Fourier transform infrared (FTIR) spectra were recorded on a Nexus 670 (Thermo Nicolet, USA) spectrometer.

Raman spectra were obtained by using a dispersive spectrophotometer Jobin-Yvon LabRam HR Evolution with 325 nm light for sample excitation and a CCD detector cooled to -70 °C. The laser power used was between 0.5 and 4 mW.

X-Ray diffraction (XRD) was performed on the Miniflx-600 (Rigaku Ltd) under ambient conditions using Cu Kα X-rays.

XPS analysis was conducted on an Axis Ultra spectrometer (Kratos Analytical Ltd) with monochromated Al Kα radiation at ca. 5×10^{-9} Pa. In order to investigate the doping ratio, we used a reported method to remove excessive F127.[1] In this method, as the result of its temperature

dependent, the micellar Pluronic F127 molecules could be converted to unimeric molecules at a temperature lower than its CMT (e.g., 4 °C). As a consequence, the unimeric F127 molecules could be easily dialyzed at 4 °C for 2 days (in water). The final product can then be collected by centrifugation (11000 rpm, 15 min).

The hydrodynamic size distributions and zeta potentials of the samples were measured using dynamic light scattering (DLS) techniques by a Zetasizer Nano instrument (Malvern, UK) at 25 °C.

The average crystallite size of PPy nanoparticles are estimated using the Scherrer equation,[2]

$$L = \frac{0.9\lambda}{\beta \cos\theta} \quad (1)$$

where β is the full width at half maximum (FWHM) of the angle of diffraction in radians, λ is the X-ray wavelength～1.54 A° and θ is the Bragg diffraction angle in degrees.

6. Photothermal Performance of NS.

To evaluate *in vitro* photothermal performance, aqueous suspensions of NS (100, 80, 50, and 20 μg mL^{-1}) were irradiated by an 808 nm NIR laser for 20 min at a power density of 0.5 W cm^{-2}. Deionized water was analyzed as control, and the corresponding temperature changes were recorded using a digital thermometer with a thermocouple probe. The photothermal stability of the NS was assessed by irradiation with an 808 nm laser (0.5 W cm^{-2}) for 20 min and subsequent cooling to room

temperature without irradiation. Three cycles of laser on/off were carried out to evaluate photostability. To determine the photothermal conversion efficiency, a suspension of the NS (50 μg mL^{-1}) was continuously irradiated under an 808 nm laser (0.5 W cm^{-2}). The laser was turned off when the temperature was stabilized, followed by the spontaneous cooling of the solution down to room temperature.

The photothermal conversion efficiency (η) of NS was determined according to the following equation used in the reported studies.

$$\eta = \frac{hS(T_{max} - T_{Surr}) - Q_0}{I(1 - 10^{-A_{808}})} \tag{2}$$

Where h is the heat transfer coefficient, S is the sample container surface area, T_{max} is the steady state maximum temperature of NS suspension under laser irradiation, T_{surr} is the ambient room temperature, Q_0 is the background energy input by the solvent and the sample container without the presence of NS, I is the laser power (0.5 W cm^{-2}), and A_{808} is the absorbance of NS (50 μg mL^{-1}) at 808 nm.

The value of hS was calculated according to the following equation:

$$\tau_s = \frac{\sum_i m_i c_{p,i}}{hS} \tag{3}$$

Where τ_s is the characteristic thermal time and m is the mass and c_p is the heat capacity of each i component of the sample cell. The mass of the

nanoplatelets suspension was 1.0 g, and its heat capacity ($c_{p,s}$) was approximated to be 4.187 J g^{-1} K^{-1} (the heat capacity of water). In addition, the mass of the quartz cuvette was 5.5 g, and its heat capacity ($c_{p,c}$) was 0.839 J g^{-1} K^{-1}. In order to obtain the τ_s, a dimensionless driving force temperature, θ is introduced using the maximum system temperature,

$$\theta = \frac{T - T_{surr}}{T_{max} - T_{surr}} \tag{4}$$

At the cooling period, τ_s could be calculated according to the following expression, where t is the time (in s):

$$t = -\tau_s ln\theta \tag{5}$$

The heat energy (Q_0) of the sample cell and solvent without nanoplatelets was calculated from an independent experiment using the following equation:

$$Q_0 = hS(T_{max} - T_{Surr}) \tag{6}$$

The photothermal conversion efficiency (η) of NS samples was calculated to be 46.35%

The band gap can be acquired by the Tauc's plot according to the following equation:[3]

$$\alpha h\nu = A(h\nu - Eg)^{1/2} \tag{7}$$

where α, ν, h, Eg, and A are absorption coefficient, light frequency, Planck's constant, band gap, and a constant, respectively.

7. PES loading and releasing experiments

PES was loaded into NS by mixing PES solution (1 mL, 1.0 mg mL^{-1}) with nanosheets (1 mg) in PBS buffer for 24 h at room temperature. The suspension was dialyzed against distilled water for 24 h. The concentration of the PES in the dialysis solution was analyzed with an UV-vis spectrophotometer (NanoDrop One 2000, Thermo) at a wavelength of 260 nm (PES). The amount of PES loaded into FeOOH–PPy NS was calculated by subtracting the mass of PES in the supernatant from the total mass of drug in the initial solution.

The release study was conducted as follows. First, 1 mg of drug-loaded nanoparticles (in dialysis bags) were dispersed in 6 mL of PBS buffer (pH 7.4) or in sodium acetate buffer solutions (20 mM, pH 5.5) with the same ionic strength (150 mM) as the PBS solution. At the predetermined time intervals, 0.5 mL of solution was withdrawn from the solution outside the dialysis bags, and the amount of released drug was analyzed by UV-vis. For keeping a constant volume, 0.5 mL of fresh medium was added after each sampling. All drug release results were averaged with three measurements. Calculation of the corrected concentration of released PES is based on the following equation:

$$Cc = Ct + \frac{V}{v}\sum_{0}^{t-1} Ct \tag{8}$$

Where Cc is the corrected concentration at time t; Ct is the apparent concentration at time t; v is the volume of sample taken (0.5 mL); and V is the total volume of the release fluid (6 mL).

8. Cell culture

For cell culture experiments, MCF-7/ADR cells were cultured in RPMI-1640 medium (GIBCO, New York) supplemented with 10% fetal bovine serum (FBS, GIBCO), 100 μg mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin and 1 μg mL^{-1} DOX. All cells were cultured at 37 °C in a humidified incubator containing 5% CO_2. The culture medium was changed every two days prior to experimental operation. For all experiments, cells were harvested by using 0.25% trypsin (Sigma) in PBS solution and resuspended in fresh medium before plating.

9. Cell uptake (confocal microscopy)

To further study the cell uptake of NS, MCF-7/ADR cells were cultured onto confocal microscopy dishes at a density of 1×10^5 cells per dish. Firstly, 200 μg of calcium was loaded into NS by adsorption in MES buffer (10 mM, pH 4). The mixture was stirred at room temperature for 2 h, and then dialysis with water for two days to collect the fluorescence labeled NS. The cells were treated with NS at a final concentration of 100 μg mL^{-1}. After incubation for 24 h, the media was removed, and the cells were washed with PBS for three times. Then, 0.2 mL of Hoechst 33258 was added, and incubated for 10 min to stain the

nuclei. At last, 1 mL of glycerin solution was added and the cells were visualized under a confocal laser scanning microscope (TCS SP8, Leica). Blue and green luminescent emissions from Hoechst 33258 and calcein were excited at the wavelength of 405 nm and 490 nm, respectively. The emission wavelengths were ranged from 425 nm to 475 nm for Hoechst 33258 and 500 to 515 nm for calcein. There was no interference between these two channels. The scanning mode was in sequential frame.

10.Cytotoxicity assay

CCK-8 assay was applied to evaluate cytotoxicity using MCF-7/ADR cells. The cells were seeded onto 96-well plates at a density of 8×10^3 cells per well and incubated at 37 °C for 24 h before the cytotoxicity assay. Afterward, the culture medium was replaced with fresh cell culture medium containing different concentrations of NS. After incubation for 24 h, the medium was removed and the wells were washed with PBS. Subsequently, 20 μL of CCK-8 together with 200 μL of fresh medium was added, and the cells were further incubated for 2 h. Finally, the absorbance of each well at 450 nm was measured using a microplate reader (Bio-Rad 680, USA).

11. Photocytotoxicity

To investigate the photocytotoxicity, the MCF-7/ADR cells were seeded onto 96-well plates at a density of 8×10^3 cells per well and incubated at 37 °C for 24 h before the cytotoxicity assay. Afterward, the

culture medium was replaced with fresh cell culture medium containing different concentrations of NS and PES loaded FeOOH–PPy NS (NS–PES). After incubation for 24 h, the medium was removed and the wells were washed with PBS. Each group was irradiated with NIR (808 nm, 0.5 W cm^{-2}) for 10 min. Then, the cells were incubated for another 30 min. Subsequently, 20 μL of CCK-8 together with 200 μL of fresh medium was added, and the cells were further incubated for 2 h. Finally, the absorbance of each well at 450 nm was measured using a microplate reader (Bio-Rad 680, USA).

To visualize NS's ability of killing cancerous cells through PTT, MCF-7/ADR cells after laser treatment were imaged after co-staining of Calcein-AM and PI. Briefly, MCF-7/ADR cells grown to 80 % into the confocal laser glass dishes were incubated with NS at a concentration of 100 μg mL^{-1} for 24 h. Then cells were irradiated by 808 nm laser at a power density of 0.5 W cm^{-2} for 10 min. Afterwards, the cell suspension was collected and washed briefly in PBS. Before incubation for an additional 0.5 h, the cells were stained with 2.0 μM CalceinAM and 2.0 μM propidium iodide (PI). Fluorescence images of cells were collected using an inverted fluorescence microscope (IX-71, Olympus).

12.Western Blot Analysis

To investigate the effect of different treatments on HSP70 expression, MCF-7/ADR cells were cultured onto six well plates at a density of 1 ×

10^5 cells per dish. Then, the cells were treated with NS–PES at the concentration of 100 μg mL^{-1}. Untreated MCF-7/ADR cells were set as control group. After incubation for 24 h, the media were removed, and the cells were washed with PBS for three times. The NS–PES group and the control group of cells only were irradiated with NIR (808 nm, 0.5 W cm^{-2}) for 10 min. By incubation for another 30 min, the cells of each group were collected and washed twice with ice-cold PBS, then added the appropriate amount of CelLytic MT reagents (Sigma-Aldrich, St. Louis, MO, USA). The samples were centrifuged at 10000 rpm for 10 min at 4 °C. The supernatant was isolated and maintained at –80 °C. Protein levels were measured using the Bradford method. Proteins were separated by polyacrylamide gel electrophoresis (PAGE) and transferred on to polyvinylidene fluoride (PVDF) membranes that were then placed in 5% skim milk powder solution at room temperature for 1 h. The caspase-3 antibody (1:1000) was added to the membranes overnight at 4 °C and incubated with antirabbit secondary antibody (1:5000) at room temperature for 2 h. Detection was performed using enhanced chemiluminescence (ECL) reagent. The β-actin expression level was used as an internal control.

B: Supplementary Figures

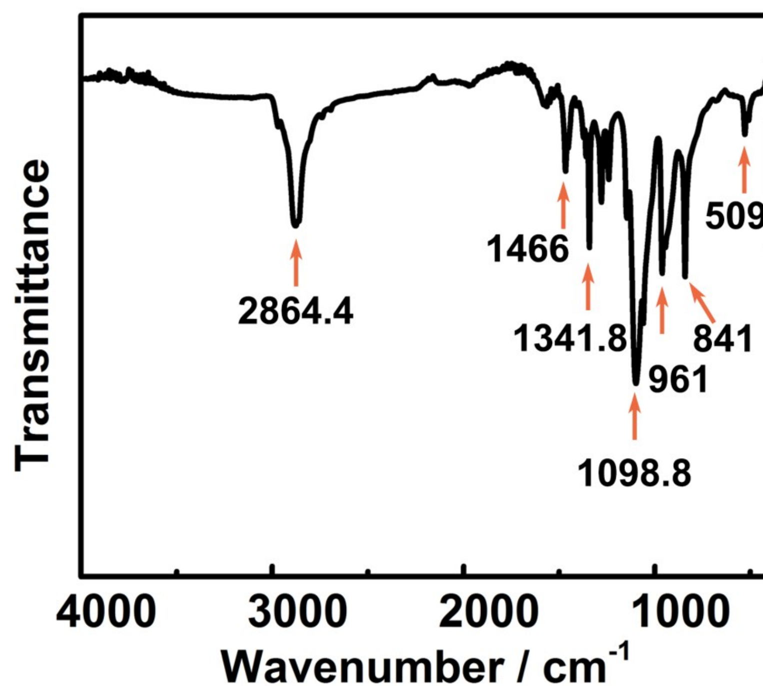

Fig. S1. FTIR spectrum of FeOOH-PPy nanosheets (NS). The FTIR was used to investigate the composition of hexagonal nanosheets. As shown in Fig S1, the skeletal band at 1098.8 cm^{-1} and the symmetric C=C stretching band at 1341.8 cm^{-1} are characteristic peaks of PPy, hydroxyl (841, cm^{-1}) and Fe-O (509 cm^{-1}) stretching vibration of FeOOH, respectively.

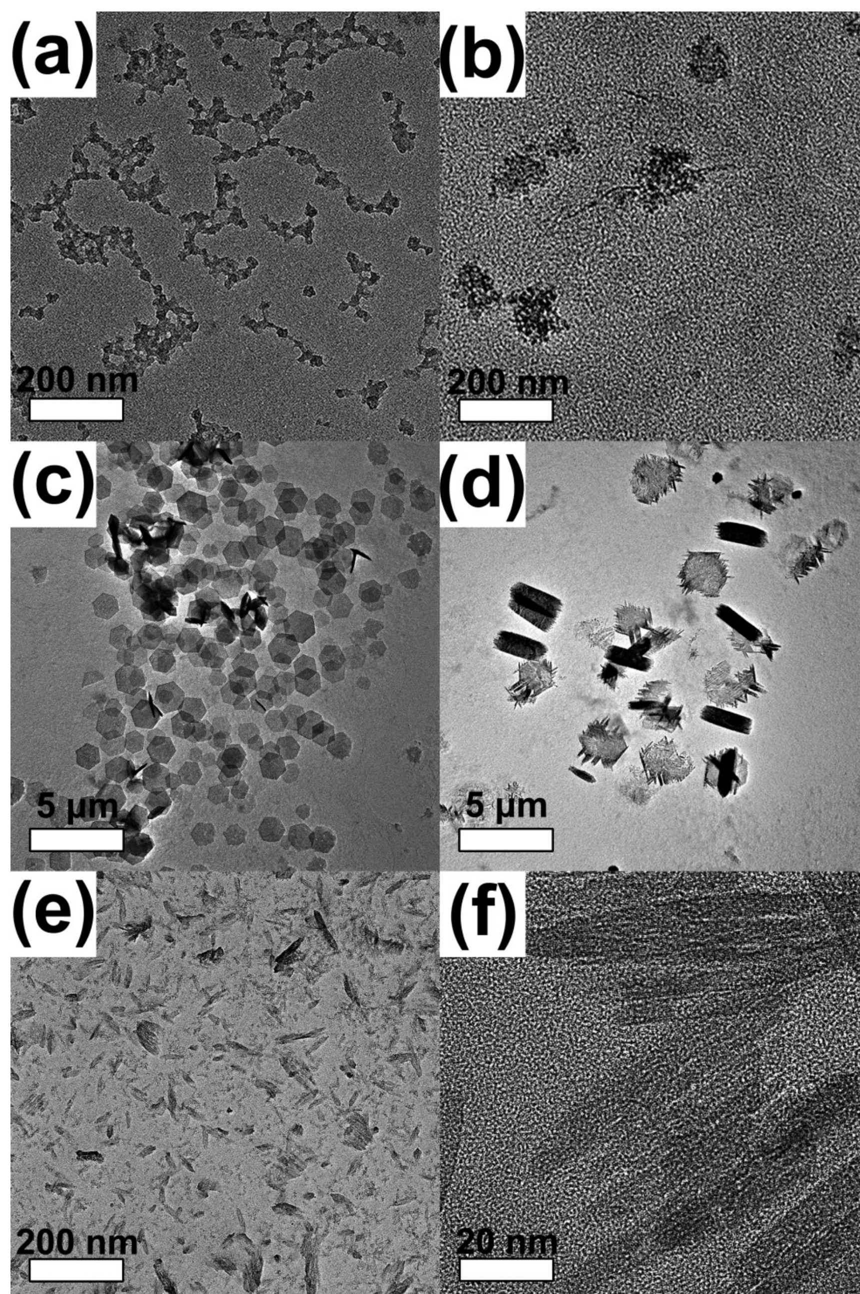

Fig. S2. TEM images of the polypyrrole (PPy) nanomaterials prepared under different molar ratios of DA/Py: 0 (a), 0.012 (b), 0.017 (c), 0.019 (d), 0.022 (e) and 0.045 (f), respectively.

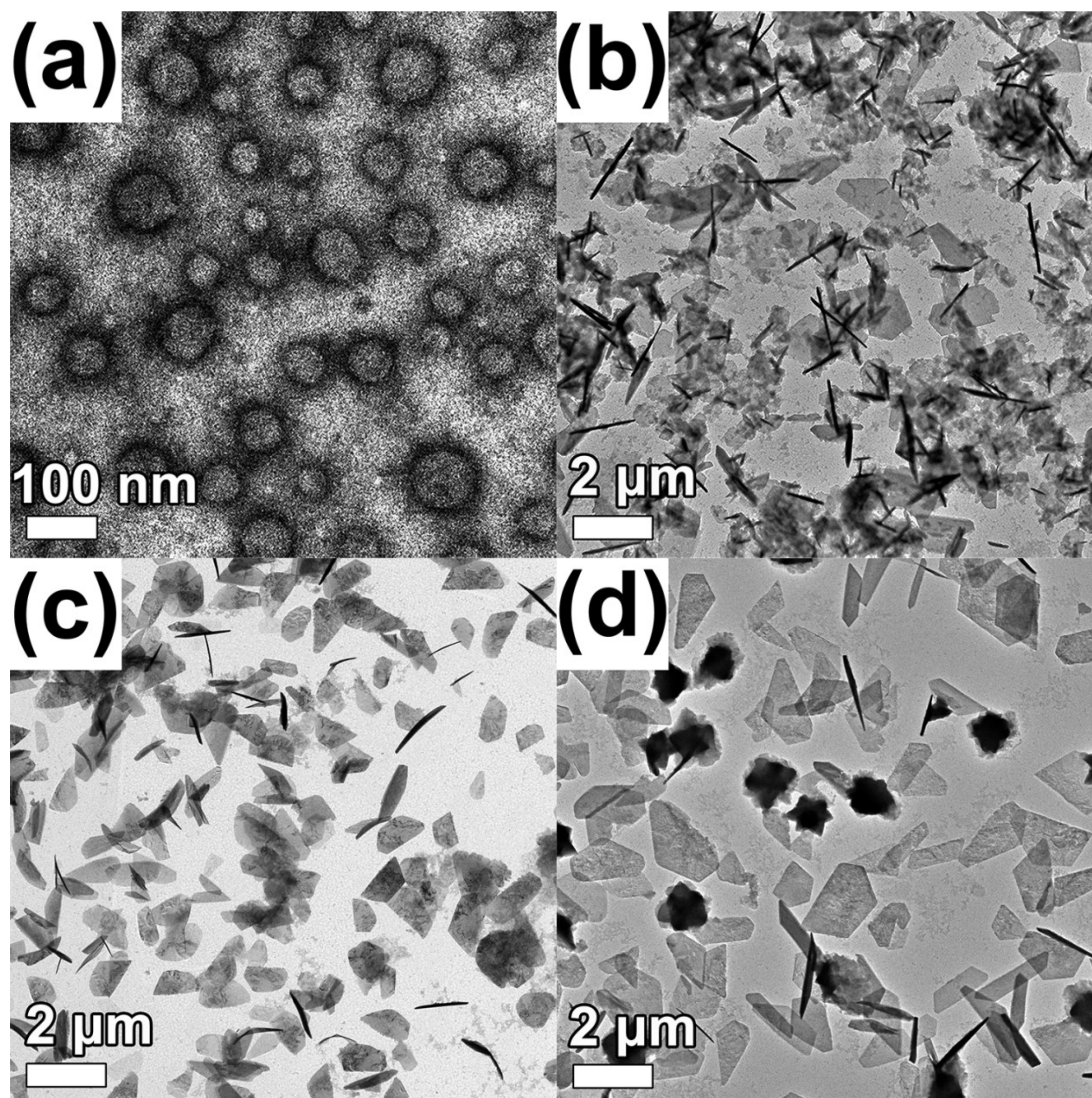

Fig. S3. TEM images of: vesicles formed by F127 and THF were negatively stained using 2% phosphotungstic acid (pH=7) before characterization. (a), NS products synthesized with altered recipes: more $FeCl_3$ (46.25 mg, b), less THF (450 μL, c) and lower temperature (4 °C, d).

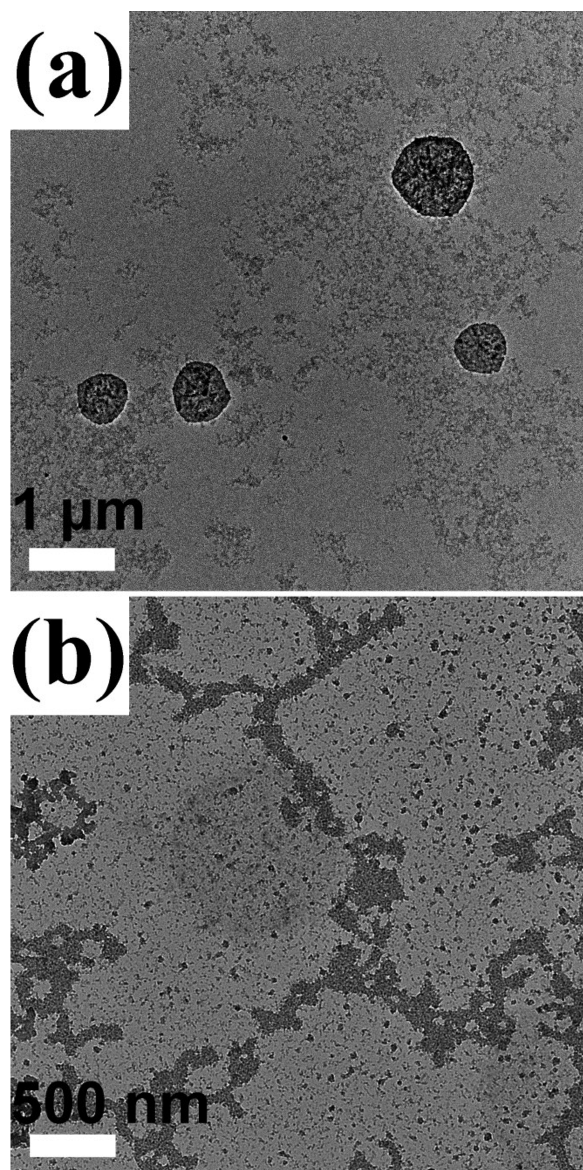

Fig. S4. TEM images of PPy nanomaterials synthesized at more F127 amounts: 150 mg (a) and 300 mg (b).

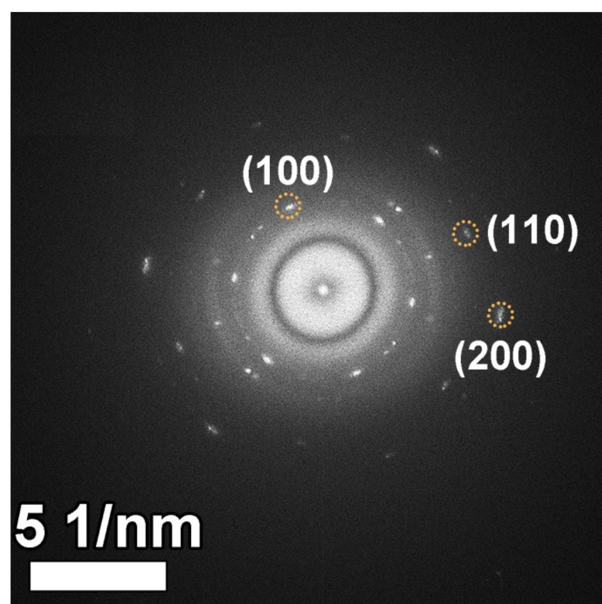

Fig. S5. Selected area electron diffraction (SAED) pattern of NS. This pattern contains three sets of spots ((100), (110), (200) facets) similar to the sixfold symmetry diffraction pattern.

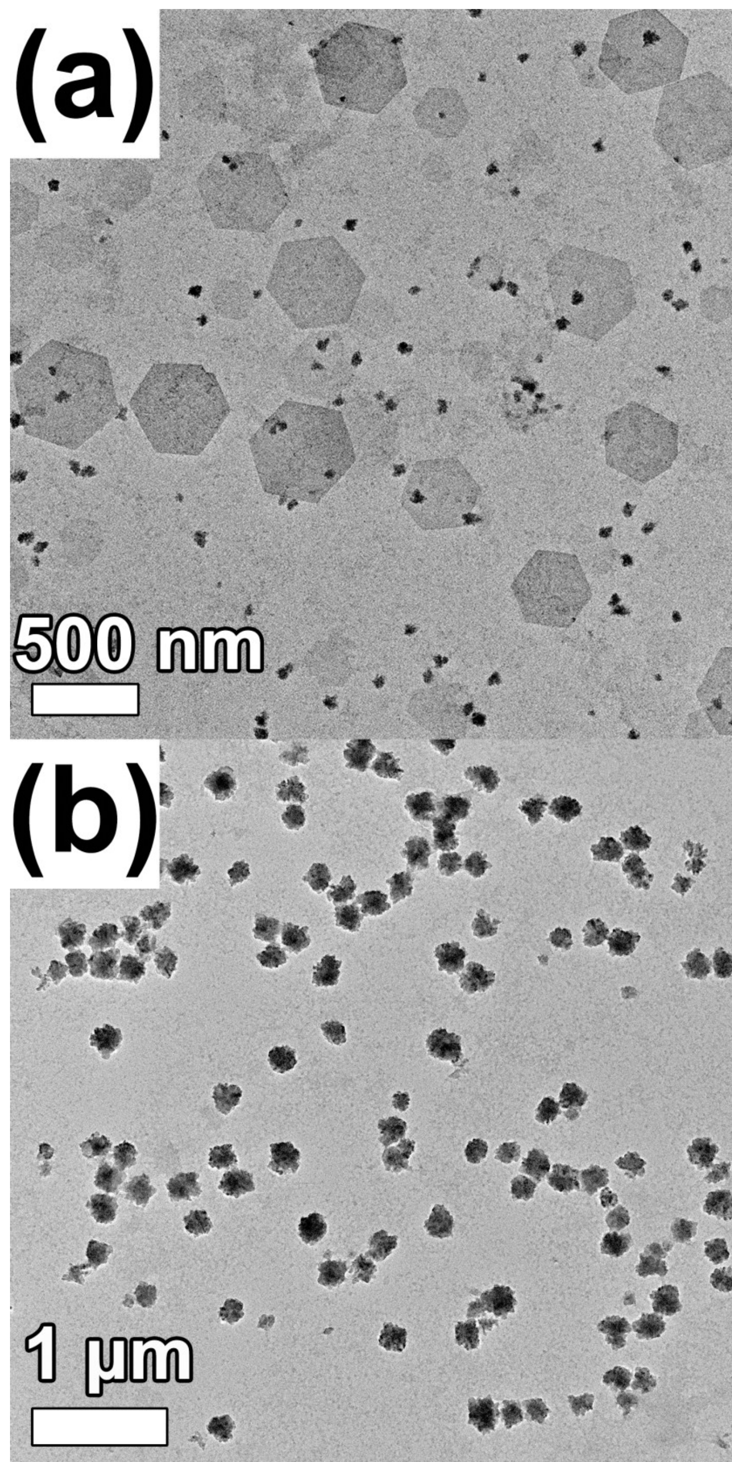

Fig. S6. TEM images of PPy nanomaterials synthesized at higher temperatures: 50 °C (a) and 60 °C (b).

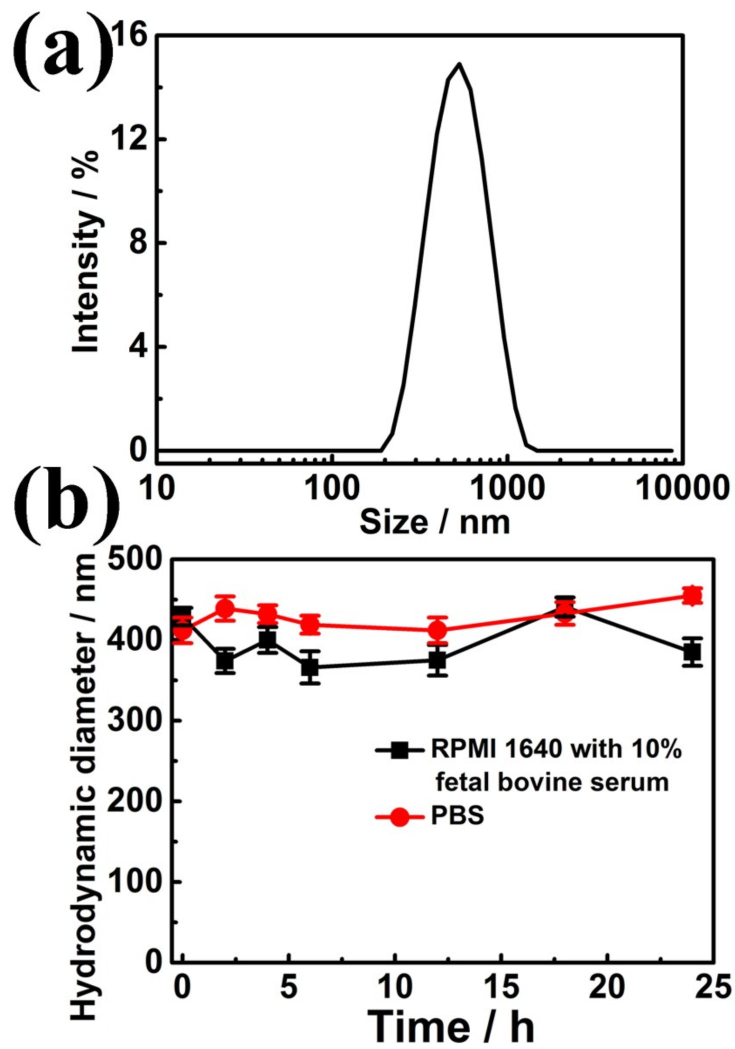

Fig. S7. Hydrodynamic size distributions data (a) and stability study (b) of NS in cell culture medium supplemented with 10% fetal bovine serum (red line) or PBS (black line) over time by monitoring the average hydrodynamic diameters.

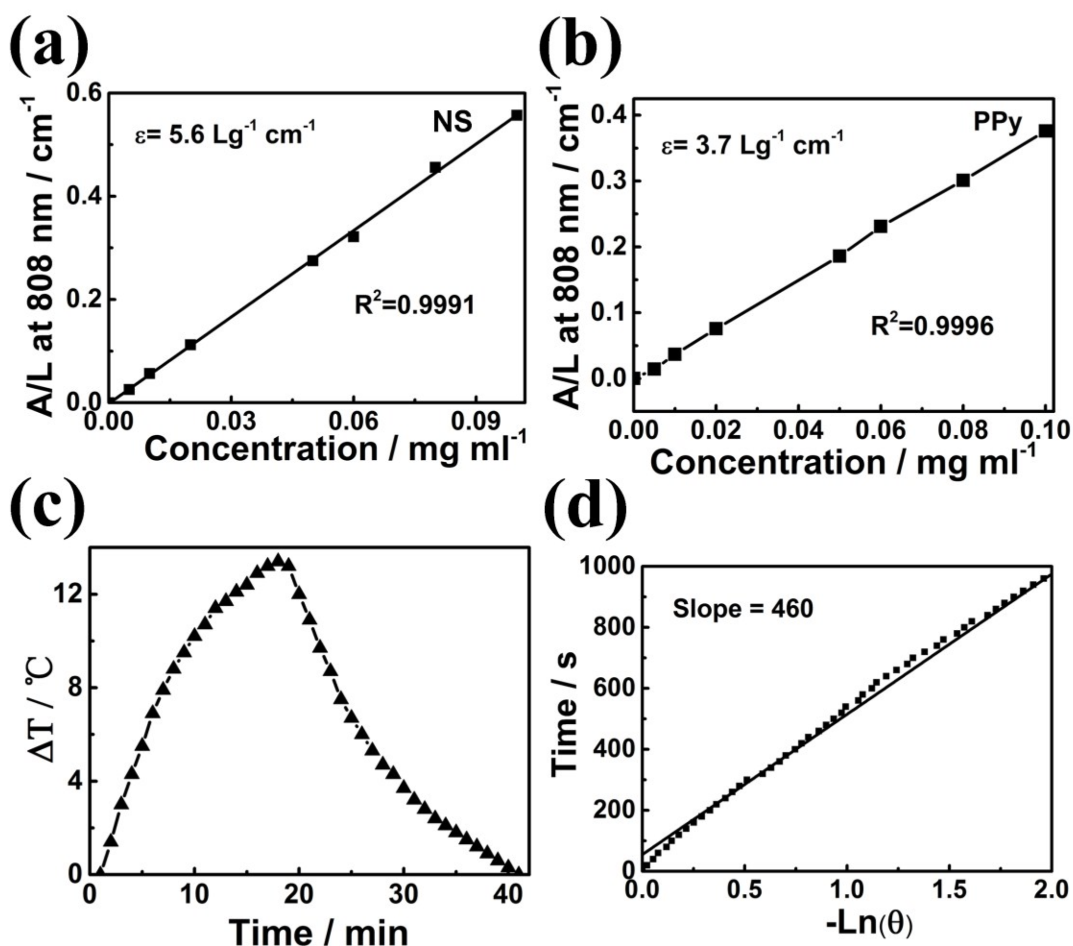

Fig. S8. Extinction coefficient curve of NS (a) and PPy nanoparticles (b) at different concentrations (0-100 μg mL^{-1}), respectively. Photothermal effect of an aqueous dispersion of PPy nanoparticles under the irradiation with a NIR laser (808 nm, 0.5 W cm^{-2}) which was turned off after 18 min (c). Plot of cooling time versus negative natural logarithm of the temperature driving force (⊿T/⊿Tmax) in the cooling stage. The thermal time constant for heat transfer from the system is determined to be τ_s = 460 s (d).

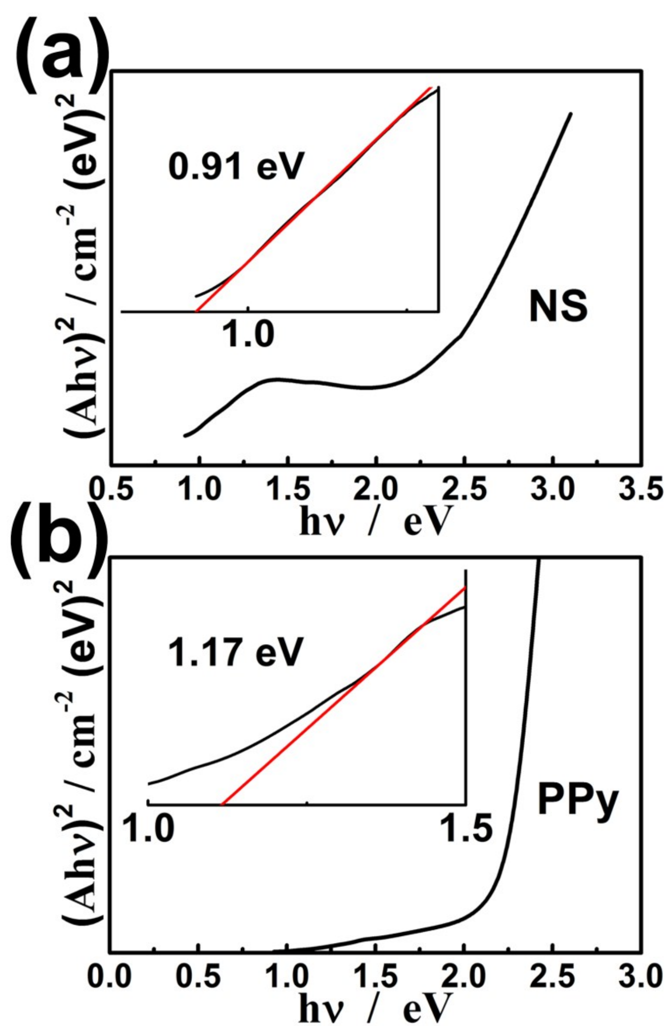

Fig. S9. Tauc's plot of $(\alpha h\nu)^2$ versus $h\nu$ for NS (a) and PPy (b) nanoparticles.

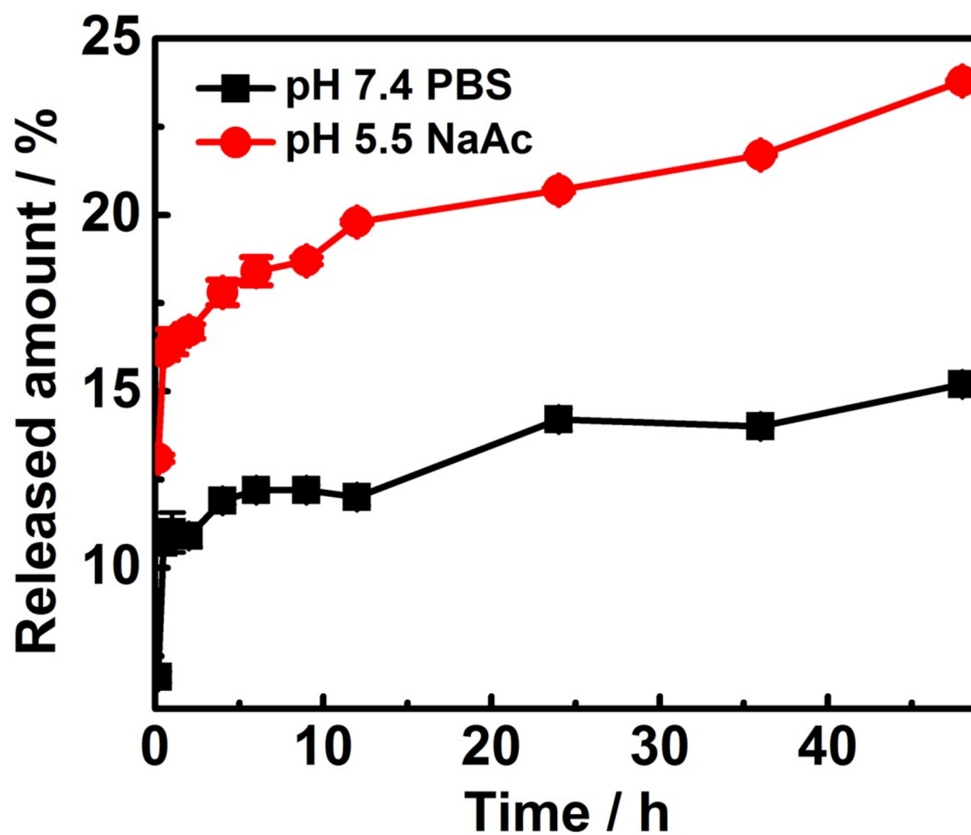

Fig. S10. PES release curve of NS at different conditions: pH 7.4 and pH 5.5.

References

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