Electronic Supplementary Material (ESI) for Nanoscale Horizons.

Champagne inspired dual chained-responsive thrombolytic drug release platform based on black phosphorus nanosheets for accelerated thrombolysis

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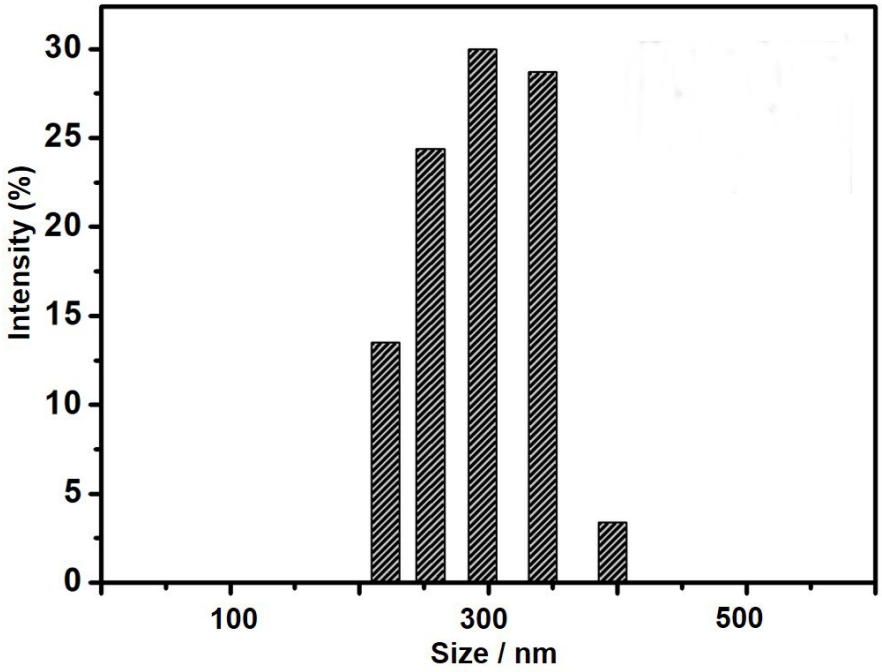
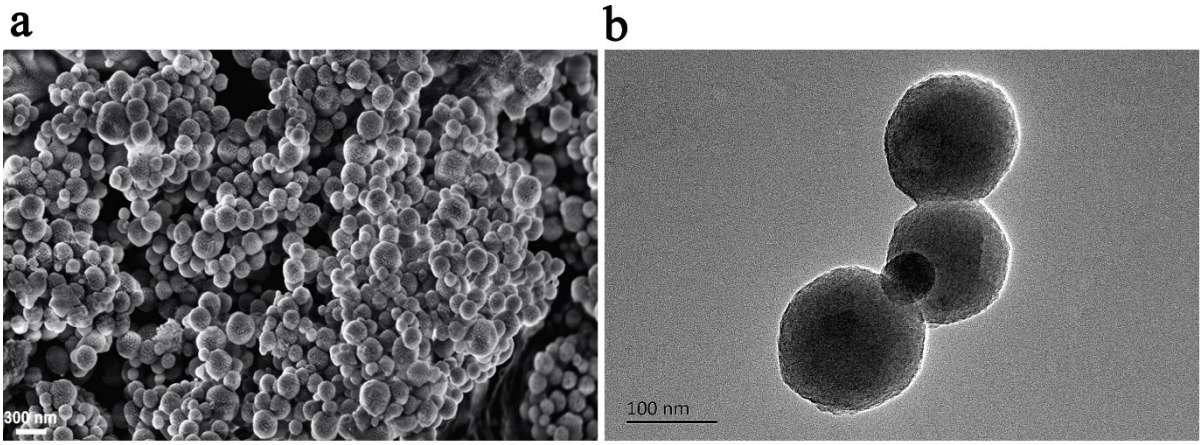
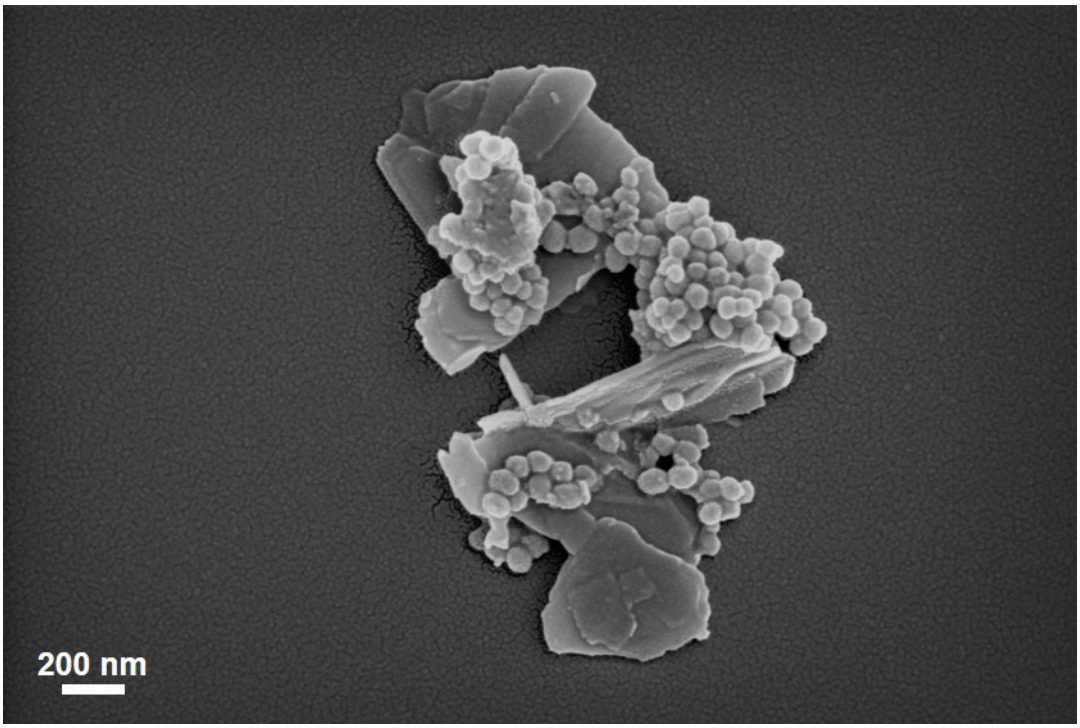
Supplementary Information



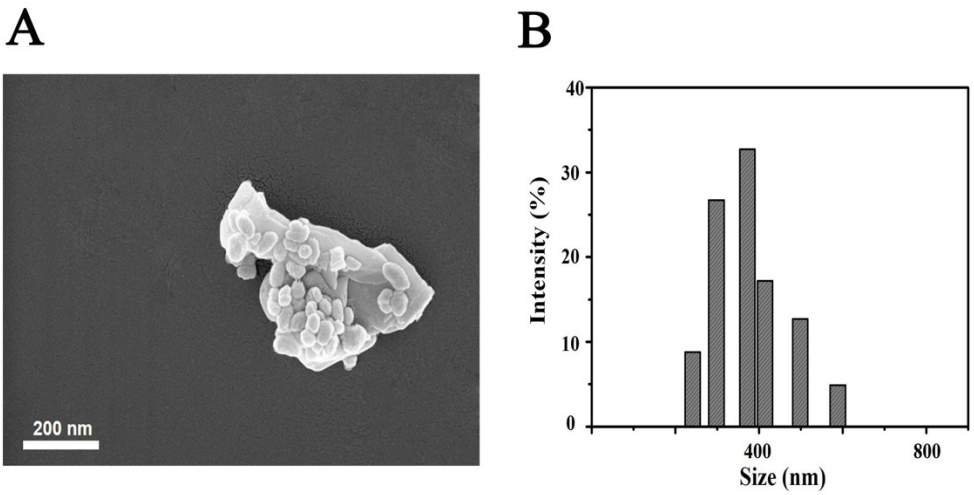
Supplementary Fig. 1 The dynamic light scattering of BPNs.



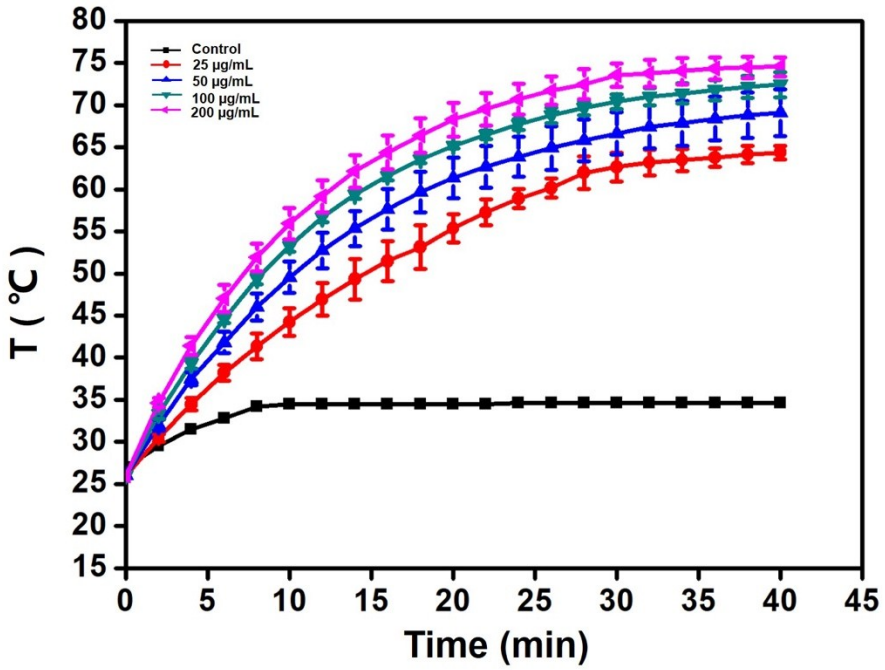
Supplementary Fig. 2 The SEM (a) and TEM (b) images of MSNs.



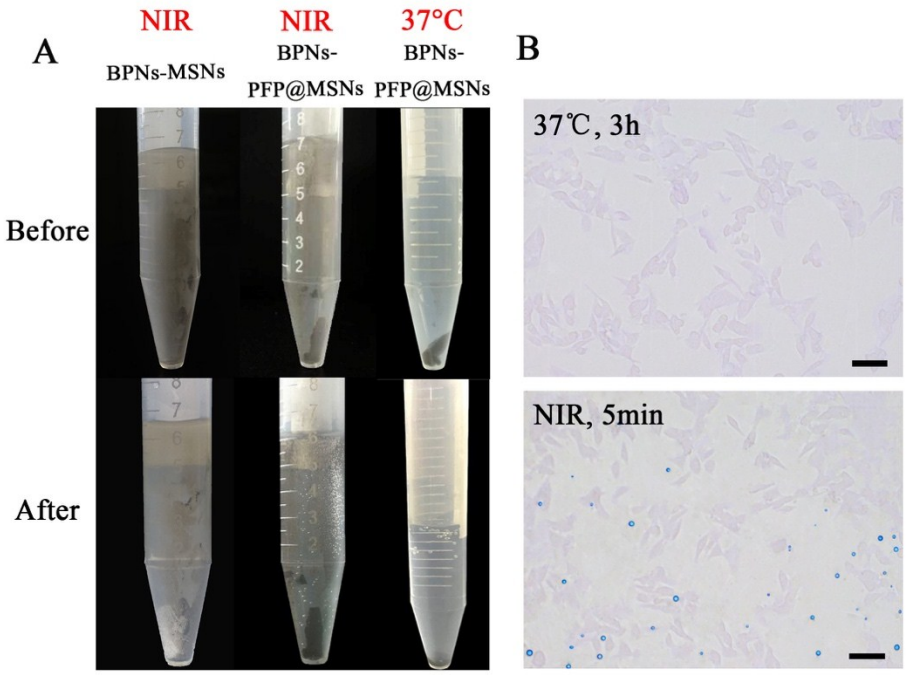
Supplementary Fig. 3 The SEM image of BPNs-MSNs placing in saline for 7 days.



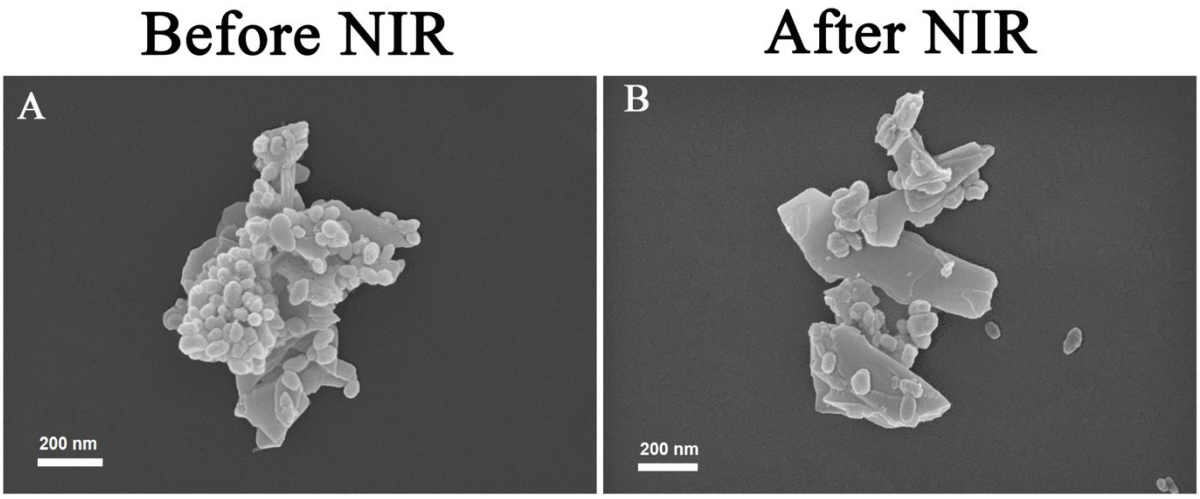
Supplementary Fig. 4 The SEM image and DLS of BPNs-MSNs placed in saline for 14 days.



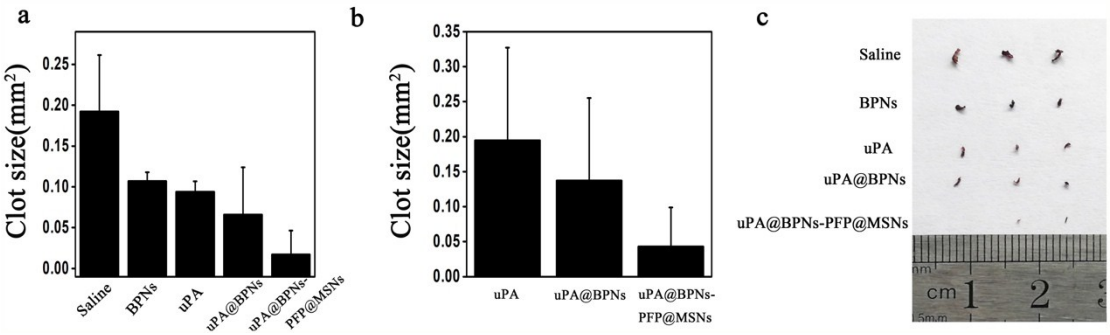
Supplementary Fig. 5. The heating curve of BPNs at a power density of 0.2 W cm^{-2}.



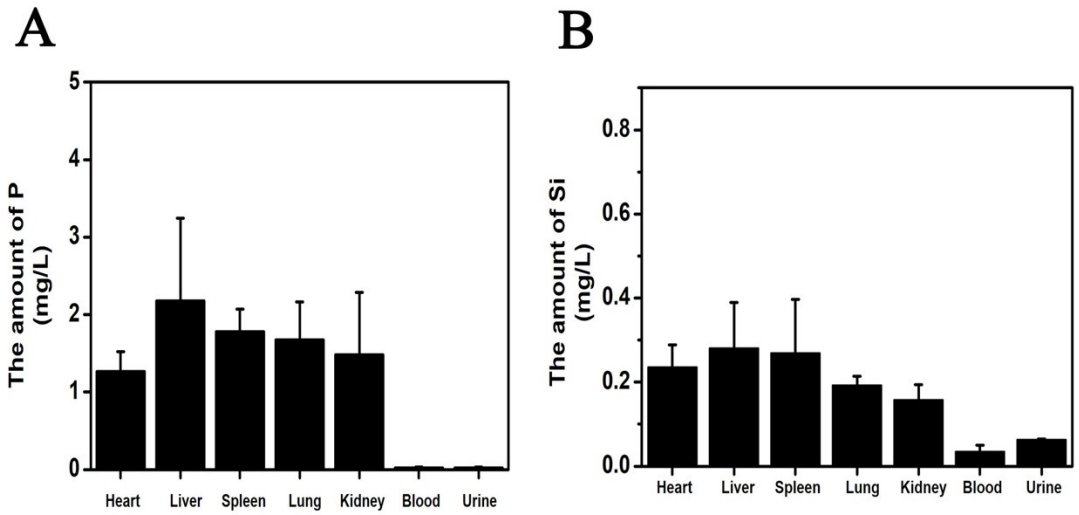
Supplementary Fig. 6 (A) The photos of generated bubbles before and after different treatments; (B) The images of generated bubbles after the BPNs-PFP@MSNs were incubated with B16 cells and treated with NIR (808 nm, 0.2 W cm^{-2}) or 37 °C.



Supplementary Fig. 7 The SEM images of BPNs-MSNs before and after NIR laser irradiation (808 nm, 0.2 W cm^{-2}, 10 min).



Supplementary Fig. 8 The clot size of thrombus after treatment for 24 h (a) and 12 h (b); (c) The photos of thrombi after treatment for 24 h.



Supplementary Fig. 9 The amount of P (A) and Si (B) were determined in the major organs, blood and urine after treatment for 7 days. Error bars represented standard deviation of three separate measurements.

Materials and methods

Materials: The Black Phosphorus (BP) crystals were purchased from HWRK Chemical Co.,Ltd. (Beijing, China) and stored in a fully dark Ar glove box. uPA, Fluorescein-isothiocyanate (FITC), Hexadecyl trimethyl ammonium bromide (CTAB), Tetraethyl orthosilicate, 1-Methyl-2-pyrrolidinone (NMP) were purchased from Makclin Biochemical Co.,Ltd. (Shanghai,China). Perfluoro-n-pentane (PFP) was obtained from Strem Chemicals Co., Ltd. (USA). The BCA protein assay kit and other Elisa assay kits were purchased from Beijing solarbio science&technology Co.,Ltd. Human Umbilical Vein Endothelial Cells (HUVEC), melanoma cell line B16 and human blood were obtained from The second Affiliated Hospital of Nanchang University (Nanchang, China). All the other related reagents and solvents were of analytical purity and used without any further purification.

Characterization: The morphology of the sample was characterized using transmission electron microscopy (TEM) and scanning electron microscopy (SEM). The size and surface charge of nanostructures was obtained with Zeta potential measurements and dynamic light scattering (DLS) test. The nanostructures' FTIR were recorded by Thermo Scientific Nicolet 5700 spectrometer. The Energy Dispersive Spectrometer (EDS) was collected on an X-ray photoelectron spectroscopy to analyze the element of the whole nano-system. Brunauer Emmett Teller analyze were recorded by Multi point BET tester. Gas Chromatography-Mass Spectrometer was used to detect the loading of PFP in the MSNs and the extractant is tri-chlorotri-fluoroethane (CFC-113). A fiber-coupled continuous semiconductor diode laser (808 nm) with the power density of 0.2 W cm^{-2} was used as the irradiated light source. Enzyme standard instrument was used to measure the absorbance of the corresponding compound.

Preparation of the Platform: We synthesized BPNs and MSNs via the previous report. FITC-uPA was obtained with the chemical synthesis method. Firstly, 10 mg uPA was weighted, and transferred into a single-mouth bottle, then 10 mL PBS was added to make the uPA solution's final concentration was 1 mg mL^{-1}. Secondly, 0.75 mL FITC methanol solution (1 mg mL^{-1}) was added, then placed the single-mouth bottle in 4 °C environment and stirred the mixture vigorously for 8 h, the light should be avoided in attention. After 8 h of reaction, the reaction was terminated by adding adequate ammonium chloride (50 mmol L^{-1}). Stirring the mixture

for another 2 h, the free FITC and FITC-uPA was separated by dialysis bag (10kDa), and the FITC-uPA solution was freeze-dried to obtain a final product (FITC-uPA).

For uPA@BPNs, 1 mg BPNs powders were dispersed in 3.5 mL PBS buffer solution, and 2 mg uPA were dispersed in 1.5 mL PBS buffer solution, and then mixed these solutions together and stirred it overnight. After stirred vigorously in dark overnight, the obtained uPA loaded BP nanosheets (uPA@BPNs) were gathered by centrifugation and washed with deionized water.

For PFP@MSNs, 2 mg MSNs powders were transferred into a one-neck flask with rubber stopper, then air inside was evacuated for 1 min followed by injection of 1 mL PFP into the flask. The whole system was ultrasonic oscillated for 5 min at the temperature of 4 °C. Then added 5 mL ice-water to the system and centrifuged it at 10000 rpm for 5 min to remove the redundant PFP. The sample was re-dispersed with 5 mL ice-water for next operation.

For BPNs-MSNs, according to the above protocol, BPNs and MSNs were prepared separately, and then joined them together by a method of stirring overnight in a dark environment.

For BPNs-PFP@MSNs, materials' monomer was synthesized separately according to the above protocol, and then joined them together by a method of stirring overnight in a dark environment and the stirring temperature was be controlled at 4 °C.

For uPA@BPNs-PFP@MSNs, according to the above protocol, uPA@BPNs and PFP@MSNs were synthesized separately, then joined them together by a method of stirring overnight in a dark environment and the stirring temperature was be controlled at 4 °C.

The stability assessment of BPNs-MSNs: 5 mL of BPNs-MSNs aqueous solution at 200 μg mL^{-1} was added into transparent quartz vial, then the system was kept peacefully with 4 °C for 14 days, the nanosheets' size was determined by dynamic light scattering (DLS) in 1day, 7 day and 14 day. What's more , the BPNs-MSNs ' morphology before and after laser irradiation was also be studied to show the stability of uPA-BPNs@PFP-MSNs.

Photothermal effect assessment of BPNs, BPNs-MSNs: 5 mL of BPNs, BPNs-MSNs aqueous solution at different concentration (0, 25, 50, 100, 200 μg mL^{-1}) was added into transparent quartz vial. A fiber-coupled continuous semiconductor diode laser (808 nm) with the power density of 0.2 W cm^{-2} was used as the light source to irradiate the bottom of the vial. A thermometer was inserted into the solution to monitor the temperature change. After

fabricating all the device, the temperature's change was recorded every 2-5 min, the whole heating up time was about 40 min. What's more, following a reported method, the photothermal conversion efficiency(PCTE) η can be determined by equation (1):

$$\eta T = (hA \cdot \Delta T_{max} - Q_s)/I.(1 - 10^{-A808}) \quad \text{Equation (1)}$$

where h is heat transfer coefficient; A is the surface area of the container; ΔT_{max} is the temperature change at the maximum steady-state temperature(10 min); Qs represents the heat loss from light absorbed by the quartz sample cell itself, and it was measured independently which it is measured independently to be 0.54.I mW; I is incident laser power (0.2 W cm^{-2}), and A_{808} is the absorbance of the sample at 808 nm(for BPNs, A_{808} is 0.498, for BPNs@MSNs, A_{808} is 0.207,). The value of hA is determined by the following equation (2):

$$\tau_s = m_{water} \cdot c_{water} / hA \quad \text{Equation (2)}$$

Where τ_s is the time constant for heat transfer of the system which was determined from the slope of the cooling curve. In addition, the is m_{water} is 5.0 g and the c_{water} is 4.2 J/g,

FITC-uPA loading efficiency and the stability assessment of platform: 1 mg BPNs powders and 1 mg FITC-uPA were fully dissolved in 3.5 mL PBS buffer solution and 1.5 mL PBS buffer solution respectively, and then joined BPNs and FITC-uPA together by a method of stirring overnight. After stirring vigorously, collecting the mixture solution and centrifugate it at 10000 rpm for 10 min. Then 0.15 mL of the resulting suspension was collected and used enzyme standard instrument detect the free FITC-uPA content. The weight of FITC-uPA was calculated by standard curve of FITC-uPA. The loading efficiency and the loading capacity of the system was calculated by the following equations.

$$\text{Drug loading efficiency (\%)} = (W_0 - W_t)/W_0 \times 100\% \quad \text{(Equation 3)}$$

$$\text{Drug loading capacity (\%)} = (W_0 - W_t)/W_{NP} \times 100\% \quad \text{(Equation 4)}$$

Where W_0 is the original weight of the FITC-uPA, W_t is the weight of the FITC-uPA in supernatant, W_{NP} is the weight of BPNs.

The stability assessment of the FITC-uPA@BPNs drug delivery system is similar. After loading with FITC-uPA, the whole system was centrifugated at 10000 rpm for 10 min, and the system was kept peacefully with 4 °C for 7 days. Then in these 7 days, 0.1 mL supernatant of this system would be collected and the absorbance of the released FITC-uPA would be detected by Enzyme standard instrument.

FITC-uPA release assessment: The FITC-uPA was loaded in the BPNs according to the above protocol (synthesis of uPA@BPNs) and the release experiment was divided into two groups (temperature-promoted and photo-promoted groups). To reduce the analytical system error, each experiment group have 3 parallel samples. For temperature-promoted group, after 2 mg FITC-uPA loaded in the 1 mg BPNs and the centrifuging operation were done, the whole system (FITC-uPA@BPNs) was treated with thermostatic water bath in 37 °C heat. Then at the desired time points, 100 μL of the supernatant was collected, the fluorescence value of FITC-uPA in the supernatant was measured on a microplate reader. For the photo-promoted group, similarity, after 2 mg FITC-uPA loaded in the 1 mg BPNs and the centrifuging operation were done, the whole system (FITC-uPA@BPNs) was treated with NIR laser (0.2 W cm^{-2}), then just as the above protocol, the desired time points' sample was collected and the fluorescence value of FITC-uPA was measured on a microplate reader. The FITC-uPA's release percentage from FITC-uPA@BPNs was calculated from the following equation.

$$\text{Cumulative release (\%)} = (I_t - I_i)/(I_\infty - I_i) \times 100\% \qquad \text{(Equation 5)}$$

where I_i is the initial absorbance value of FITC-uPA without special treating, I_t is the absorbance value of FITC-uPA after special treating, I_∞ is the absorbance value of the loaded FITC-uPA.

The pulsed drug release assessment: Similarity to the above protocol, after 2 mg FITC-uPA loaded in the 1 mg BPNs, the whole system (FITC-uPA@BPNs) was treated with NIR laser (0.2 W cm^{-2}) for 2 min and without NIR laser for another 2 min, then just as the above protocol, the desired time points' sample was collected and the absorbance value of FITC-uPA was measured on a microplate reader.

Bubbles generation assessment in vitro: After BPNs-MSNs, BPNs-PFP@MSNs, was prepared, storing it into a centrifuge tube and soaking it in a thermostatic water bath at 42 °C for 10 min to observe the generation of bubbles induced by heating. 6-7 mL BPNs@MSNs, BPNs-PFP@MSNs solutions were transferred into 14 mL centrifuge tube respectively, and then exposed to a NIR laser at the power intensity of 0.2 W cm^{-2}, the radiation lasted for 10 min. B16 cells were plated into 6 cm dishes at a density of 5×10^4 cells per well and incubated overnight. After cultured in medium containing BPNs-PFP@MSNs at 25 °C with 5% CO_2 for 3

h, cells were exposed to NIR laser at the power intensity of 0.2 W cm^{-2}. The radiation lasted for 10 min and an optical microscope was used to observe the generation of microbubbles. The untreated cells were set as the control group.

Hemolysis assay: To get the pure red blood cells (RBCs), the mixture containing 1 mL human blood and 2 mL PBS solution was centrifuged at 1500 rpm for 15 min. PBS solution was used to wash the RBCs, and this operation was repeated five times. Then mixed the 0.30 mL pure red blood cells (2%) suspension and 0.30 mL sample (deionized water, PBS, BPNs-MSNs) at different concentration (0, 25, 50, 100, 200 μg mL^{-1}) together, RBCs treated with deionized water and PBS solution were set as positive and negative controls. The mixture was incubated at 37 °C for 6 h. After those operations, the mixture was centrifuged to collected the suspension, and then used the Enzyme standard instrument to measure the absorbance of the supernatants at 540 nm. The Hemolysis (%) was calculated by the following equation.

$$\text{Hemolysis}(\%) = (A_s - A_N)/(A_p - A_N) \times 100\% \quad \text{(Equation 6)}$$

Where A_s is the absorbance of sample, A_N is the absorbance of negative control, and A_p is the absorbance of positive control.

Cell culture and cytotoxicity assay: HUVEC were seeded at 5×10^3 cells per well in a 96-well microtiter plate and cultured with RPMI 1640 medium containing 10% fetal bovine serum (FBS, Gibco), 100 U mL^{-1} of penicillin and 100 μg mL^{-1} streptomycin at 37 °C under 5% CO_2 atmosphere for 24 h. Cytotoxicity assay of LDH was performed using the CytoTox 96 nonradioactive cytotoxicity assay kit respectively. Briefly, after the treating HUVECs (5000/well) in 96-well plates with BPNs-MSNs (0, 25, 50, 100, 200 μg mL^{-1}) for 24 h, the culture medium was collected, centrifuged the culture medium at 2000 rpm for 20 min, collected the supernatants. LDH release content were monitored by the microplate reader by the means of measuring the absorbance of the supernatants at 450 nm. Cytotoxicity was expressed relative to basal LDH release in untreated control cells.

Cell viability assay: BPNs-MSNs cytotoxicity were determined by CCK8 assay and Live/Dead staining assay. For CCK8 assay, firstly, HUVECs cells were seeded into the 96-well plates at the density of 2×10^3 cells per well and cultured it for 24 h. Then the medium was replaced with 100 μL of fresh medium containing different concentrations of BPNs-MSNs (0, 25, 50, 100 and

200 μg mL^{-1}). After incubation for 24 h, 48 h, 72 h, the medium was replaced by CCK8 solution in medium (V_{CCK8}:V_{medium}=1:9) and incubated for another 2 h. The absorbance at 450 nm (OD_{450nm}) was measured on a microplate reader.

For Live/Dead staining assay, HUVECs Cells were seeded into the 48-well plates at the density of 2 × 10^4 cells per milliliter and cultured for 12 h. Then the medium was replaced with 200 μL of fresh medium containing different concentrations of BPNs-MSNs (25, 50, 100 and 200 μg mL^{-1}). After incubation for 24 h, the cell culture medium was replaced with 5 μL Calcein-AM (5 μg mL^{-1}) and PI (10 μg mL^{-1}). Then cultured for another 30 min, the cells were washed with PBS and imaged using an inverted fluorescence microscope.

Blood Collection and Clotting: The blood clot was prepared via a method that was improved by previously reported method. KM mice (200-300 g) were narcotized with chloral hydrate (7%), and then 3 mL blood was obtained from the heart and aliquoted it into several transparent quartz vials, which contained 1 mL thrombin solution. The vials were placed at 37 °C for 3 h and then were moved to 4 °C for another 3 days.

Thrombolysis test in vitro: The prepared blood clots (92.2 mg) were placed into a transparent quartz vials containing 5 mL saline, then 1 mg sample (BPNs，uPA，uPA@BPNs，uPA@BPNs-PFP@MSNs) was added into the solution respectively, the vials were exposed to the NIR laser (0.2 W cm^{-2}) respectively. In the end, the blood clots were taken out, then photoed and weighted at the setting time.

Establishment of murine acute right jugular vein thrombosis model: All the experimental animals were treated with surgical procedures and post-operative cares which were in accordance with institutional guidelines on animal care. First, male SD rats (body weight = 200-250 g, age = 8-12 weeks) were anesthetized with a mixture of intraperitoneal ketamine/xylazine and the hair on each mouse's right below the neck was removed by the animal shaver. Secondly, the right main jugular vein of the mouse was exposed by blunt dissection methods. Thirdly, a filter paper (1 × 5 mm) soaked with 3.5% $FeCl_3$ was placed on the vessel and incubated for 10 min. In order to ensure the incubated location of the $FeCl_3$ was only placed on the surface of the vessel, another filter paper was used to remove the extra and permeated $FeCl_3$ liquid. After removal of the 3.5% $FeCl_3$ filter paper, the residual

$FeCl_3$ on the vessel was washed by 0.9% NaCl solution.

Thrombolysis test in vivo: Mice were randomly divided into 5 groups (n = 6 for each group). Including control group, BPNs group, uPA group, uPA@BPNs group, uPA@BPNs-PFP@MSNs group. After the mice model with acute right jugular vein thrombosis was established, mice were tail-vein-injected with the corresponding experimental drugs (BPNs, uPA, uPA@BPNs, uPA@BPNs-PFP@MSNs) at corresponding volume. All the administration groups' uPA content should be controlled at 0.12 mg. 10 min after injection, all of the mice's right jugular vein were irradiated with NIR laser for 10 min. Then stitching the wound in time. Re-dissecting and observing the right jugular vein after treated it for 12 h or 24 h. At the desired time, the thrombolysis of right jugular vein was photoed and the vein was separated and stored in 4% formalin solution at 4 °C for 48 h for the next histology staining to compare the thrombolysis of thrombus. What's more, 3 mL heart blood was obtained from the mice to test the index contents related to thrombolysis (D-Dimed and Fibrinogen Degradation Product).

Biodistribution and biosafety evaluation test: 15 mice were used for the toxicological test. The mice were divided into 5 groups (Saline, BPNs, uPA, uPA@BPNs, uPA@BPNs-PFP@MSNs). Then experimental dose of drug was tail-vein-injected into the mice for 3 days, after feeding for another 7 days, all the mice were euthanized and sacrificed. Heart, liver, spleen, lung and kidney of the mice were separated and stored in 4% formalin solution at 4 °C for 48 h for the next histology staining to evaluate the in vivo toxicity of us experimental drug in mice.

What's more, another 2 groups mice(n=3) were used for the ICP-MS test, experimental dose of BPNs-MSNs was tail-vein-injected into the mice, then after feeding for 24 h and 7 days, the mice were euthanized and sacrificed. Heart, liver, spleen, lung, kidney, blood and urine of the mice were collected for the next ICP-MS test to analyze the P and Si's biodistribution and biosafety in organs according to a previously report.

Statistical analysis: All experiments data were obtained at least in triplicate (n≥3). The results were expressed as mean values ± standard deviation and analyzed with Student-Newman-Keuls (SNK) test to evaluate the significance of differences between different groups. A probability value (p-value) of <0.05 was considered statistically significant (*$p < 0.05$, ** $p < 0.01$ and ***$p < 0.001$).

Ethical approval: The ethical approval is obtained from the ethics committee of Nanchang University. All animal procedures are performed according to the protocol approved by the Institutional Animal Care and Use Committee at Institute (IACUC) of Translational Medicine, Nanchang University (Grant No.2018NC-012-06), and the IACUC registration number of Institute of Translational Medicine of Nanchang University is SYXK 2018-0006.