Supplementary Information (SI) for Nanoscale.
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Supporting Information

Fibrin-targeted and pH-responsive liposomes for synergistic thrombolysis and thrombotic microenvironment reprogramming

Xinyuan Li [a #], Lingying Chen [a #], Rui Sun [a], Lishan Xiong [a], Jinhan Yan [a], Qiongfan Liu [a], Chunrong Yang [a *], Zuowen Zhang [b *], Chenhui Wang [a c *]

[a] *Chongqing Key Laboratory of Natural Product Synthesis and Drug Research, School of Pharmaceutical Sciences, Chongqing University, 55 South Daxuecheng Road, Chongqing 401331, China*

[b] *Department of Neurology, Chongqing University Jiangjin Hospital, Chongqing, China*

[c] *Key Laboratory for Molecular Enzymology and Engineering of Ministry of Education, School of Life Sciences, Jilin University, Changchun,130012, China*

[#] These authors contributed equally to this work.

*Corresponding author.

E-mail address: chryang@cqu.edu.cn; zw_zhang@cqu.edu.cn; wangchenhui@cqu.edu.cn

Experimental section:

Materials. Dioleoylphosphatidylethanolamine (DOPE) was purchased from Adamas Reagent Co., Ltd. (China). 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-(poly (ethylene glycol))$_{2k}$ (DSPE-PEG$_{2k}$), and DSPE-PEG$_{2k}$-Mal were purchased from Weihua Biological Co., Ltd. (China). CREKA (Cys-Arg-Glu-Lys-Ala) peptide was synthesized by Taopu Biological Technology Co., Ltd. (China). Cholesteryl hemisuccinate (CHEMS) was purchased from Maclin Reagent Co., Ltd. (China). Acetylsalicylic acid (ASA) was purchased from Aladdin Biochemical Technology Co. Ltd. (China). Urokinase-type tissue plasminogen activator (uPA, ST2075), the activity assay kit of uPA, the living or dead cell assay kit, Coomassie blue super fast staining solution, and Calcein-AM were acquired from Beyotime Biochemical Technology Co., Ltd. (China).

Preparation of tLipo@UA

CREKA peptide and DSPE-PEG$_{2k}$-Mal were dissolved in DMF at a molar ratio of 1.1:1. The reaction was maintained under the protection of N_2 at room temperature for 12 h with moderate stirring. The final product was dialyzed (MW 2000) against deionized water for 48 h to remove unreacted peptides. After lyophilization, DSPE-PEG$_{2k}$-CREKA was obtained.

The thin film hydration method was utilized to prepare CREKA-modified liposomes. DOPE, CHEMS, DSPE-PEG$_{2k}$/DSPE-PEG$_{2k}$-CREKA (mole ratio 10:10:1) were dissolved in chloroform in a round-bottom flask. The organic solvent was removed through rotary evaporation, and then phosphate buffer solution (PBS, pH 7.4) was

added to rehydrate the lipid film in a water bath sonicator. For the loading of uPA, the PBS solution of urokinase (50,000 U/ml) was used with the same method described above. Not encapsulated uPA was removed via ultra-high speed centrifugation at 100,000 g. ASA was added before the formation of the lipid membrane.

Characterization of tLipo@UA

Transmission electron microscopy (Talos F200S, Thermo Fisher Scientific, USA) was utilized to obtain morphology information of liposomes. The particle size and zeta potential were detected using a multi-angle particle size and zeta potential analyzer (Omini, Brookhaven, USA). The successful preparation of DSPE-PEG$_{2k}$-CREKA was confirmed via ^{1}H NMR spectroscopy (Agilent, 400 MHz, USA), and the NMR data were analyzed by MestReC.4.9.9.9 software. The successful loading of ASA was confirmed via ultraviolet-visible spectrophotometer (UV, Agilent, Cary 60, USA). The successful loading of uPA was confirmed via SDS-PAGE and Coomassie blue staining.

Evaluation of loading efficiency

Drug loading rate (LE%) of tLipo@UA was measured. After centrifuging the resultant supernatant, the absorbance was calculated. The LE% was calculated using the suggested procedure. As previously mentioned, the UV and uPA enzyme activity assay kit were used to evaluate LE% of ASA and uPA. For determining LE%, the following formula was used:

$$LE(\%) = \frac{m_{drug}}{m_{drug} + m_{lipo}} \times 100\%$$

While m_{drug} indicated the amount of ASA and uPA loaded in the nanocarriers, m_{lipo} represented the total amount of liposomes.

***In vitro* drug release rate of tLipo@UA**

To evaluate the release profile of ASA and uPA, tLipo@UA was dissolved in BSA (1 mL, pH 7.4 and pH 6.5) and centrifuged at 5000 rpm. The concentrations of ASA and uPA in the liposome solution were detected using samples taken from the supernatant every 30 min. And the amounts of ASA and uPA released were measured via UV and an enzyme activity assay kit. Based on the following equation, the computation was done:

$$\text{Drug release}(\%) = \frac{m_{\text{drug}\ release}}{m_{\text{drug}\ actual\ loading}} \times 100\%$$

Drug actual loading corresponds to the overall amount of drugs loaded into liposomes, whereas $m_{\text{drug release}}$ exhibits the amount of uPA and ASA released from liposomes.

***In vitro* thrombolytic effect of tLipo@UA**

The thrombolytic effect of tLipo@UA was determined by agar with fibrinogen and thrombin plate assay. Fibrinogen solution was prepared in 25 mL PBS buffer at a concentration of 2 mg/mL and kept at 37°C. In a separate beaker, 200 mg agar was melted and mixed with the fibrinogen solution and stirred for 1 min. The fibrinogen/agar solution was then transferred to a transparent rectangular plastic plate and spread carefully throughout the surface. A solidified fibrin gel developed after 2 h incubation at 37°C. Sample wells (3 mm diameter) were created on which 5 μL of 1 mg/mL of plasminogen was added. PBS, uPA, and tLipo@UA (pH 7.4 and pH 6.5) were added to respective wells. The plate was then incubated overnight at 37°C for complete fibrinolysis. The area of the zone was calculated to evaluate the fibrinolytic activity.

***In vitro* anti-inflammation effect of tLipo@UA**

To verify the therapeutic efficacy, the anti-inflammatory properties of the therapeutic system at the cellular level were investigated. Briefly, RAW264.7 cells were seeded and cultured for 24 h. Lipopolysaccharide (LPS, 1 μg/mL) and correspondingly administered agents (PBS, ASA, Lipo, and tLipo@UA) were added into the cells for 12 h of incubation. The concentrations of relative proinflammatory cytokines such as TNF-αand IL-6 were assayed by an enzyme-linked immunosorbent assay (ELISA).

Evaluation of fibrin targeting *in vitro*

To investigate the fibrin-targeting property of tLipo@UA, 500 μL fibrinogen (10 mg/mL) was added to 24-well plates and incubated at 37 °C for 30 min. After washing with PBS 3 times, the PBS, FITC-labeled uPA, or tLipo@UA were then added to the wells, respectively, and incubated at 37 °C for another 60 min. The wells were washed intensively, and the fluorescence of FITC was monitored and quantified via the *in vivo* small animal imaging (IVIS Lumina III, Perkin Elmer, USA).

Evaluation of thrombus targeting and biodistribution assessment *in vivo*

A thrombus model was established in the common carotid artery via 20% $FeCl_3$ solution. After exposure the common carotid artery of KM mice, the filter paper with 20% $FeCl_3$ was lay over the artery. Cy5.5-uPA and Cy5.5-tLipo@UA were injected to assess the extent of fluorescence aggregation in the carotid artery at 30 min. The intensity of fluorescence at the thrombus site was detected and quantified via IVIS.

After tail vein injection of Cy5.5-uPA and Cy5.5-tLipo@UA in 1h and 3h, the fluorescence intensities of the mice's primary organs, including the heart, liver, spleen,

lung and kidney, were examined and quantified via IVIS after dissecting the mice.

Evaluation of the thrombolytic and anti-inflammatory effect *in vivo*

The mouse black tail thrombosis model was established. The Kunming mice (KM, female, 6-7 weeks) were divided randomly into 5 groups and intravenously injected with PBS, uPA, U+A, Lipo@UA and tLipo@UA via tail veins every other day for a total of four injections, respectively (n=5). The tail length of the tails was measured daily. After 7 days, the mice were sacrificed. The tails were clipped off and collected into 4% paraformaldehyde and examined by H&E to evaluate the thrombus treatment effect.

Cell lines and animals

The brain-derived endothelial cell line bEend.3, the mouse macrophage cell line RAW264.7, and the human umbilical vein endothelial cell line HUVEC were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China).

Female KM (weight range: 20-26 g) were purchased from Ensiweier Co., Ltd. All the animal protocols followed the Animal Laboratory Ethics Committee of Chongqing University. All of them were kept under standard specific pathogen-free conditions in the animal house of Chongqing University. All the experiments related to animals were carried out according to the guidelines approved by the Laboratory Animal Welfare and Ethics Committee of Chongqing University (CQU-IACUC-RE-202308-008).

Biocompatibility of tLipo@UA

For the *in vitro* hemolysis evaluation, the collected KM mice RBCs (50 μL) were dispersed in PBS (1 mL) to obtain an erythrocyte suspension. tLipo@UA at the same

concentrations (0, 12.5, 25, 50, and 100 μg/mL) and 1% triton were incubated with the erythrocyte suspension for 3 h at 37 °C. After centrifugation (3000 rpm, 5 min), the supernatant was collected, and the absorbance of hemoglobin was detected. Moreover, for the *in vitro* cytotoxicity assays, HUVEC and bEnd.3 (8000 cells/well) were seeded into 96-well plates and incubated for 12 h. And then different concentrations (0, 12.5, 25, 50, and 100 μM) of tLipo@UA were added. Thereafter, 25 μL of MTT (5 mg/mL in PBS) was added to the culture medium and further incubated for 4 h at 37 ° C. Finally, the supernatant was discarded and replaced with 200 μL of dimethyl sulfoxide, and the absorbance at 490 nm was measured using a microplate reader (n = 3) (SpectraMax i3x, Molecular Devices, USA). Furthermore, *in vivo* biosafety of tLipo@UA was also evaluated. The PBS, uPA + ASA, Lipo@UA, tLipo@UA, and uPA (8000 U/kg) were intravenously injected into the healthy KM mice, respectively. 7 days later, the mice were sacrificed, and the plasma and main tissues were harvested. The plasma levels of ALT, AST, and BUN were measured by the ELISA. Moreover, the major organ tissues (heart, liver, spleen, lung, and kidney) were collected to stain by H&E to evaluate the pathological changes.

Statistical analysis

All results were shown as mean ± standard deviation (SD). Statistics were analyzed by GraphPad Prism 10.1.2, and differences between groups were compared by one-way analysis of variance (ANOVA). $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, and $^{****}P \leq 0.0001$.

Supporting figures:

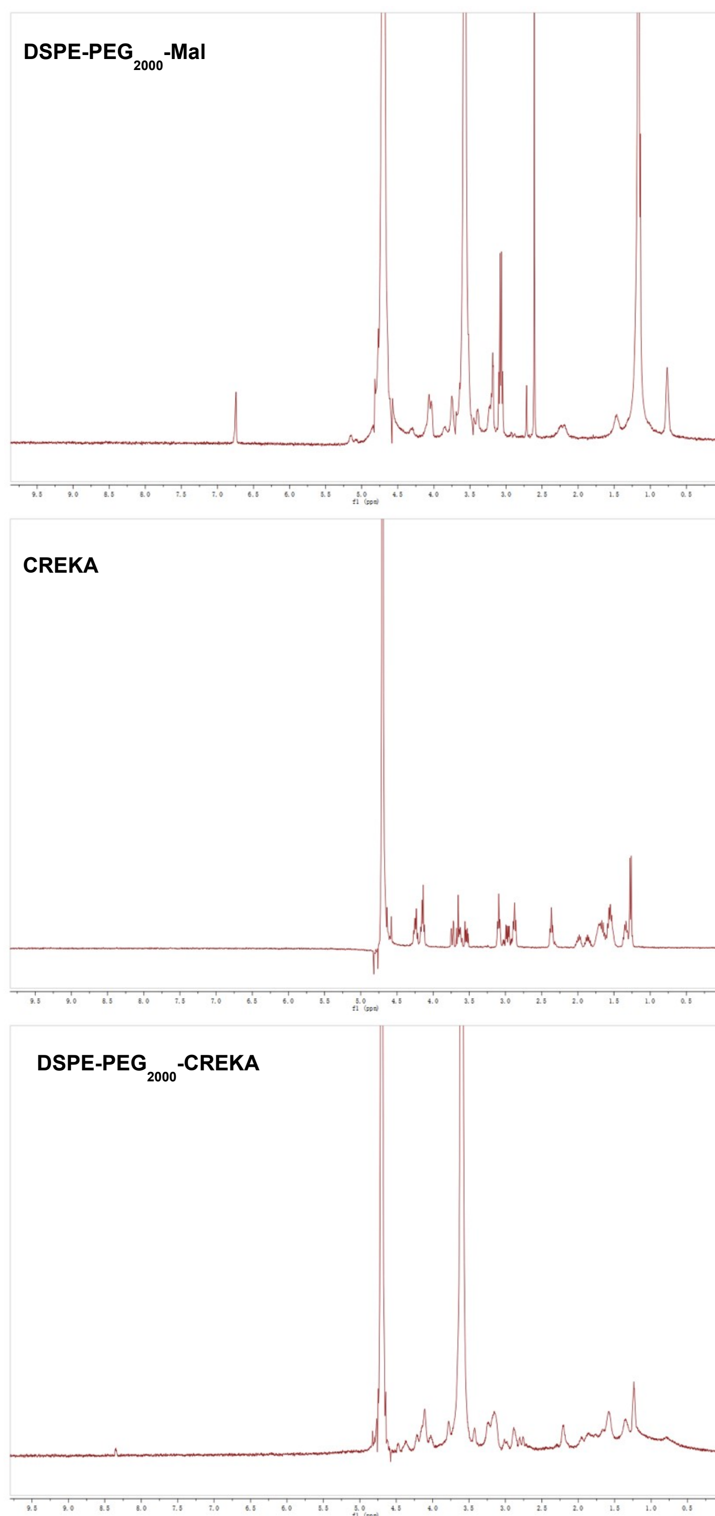

Fig. S1 ^{1}H NMR results of DSPE-PEG$_{2000}$-Mal, CREKA, and DSPE-PEG$_{2000}$-CREKA.

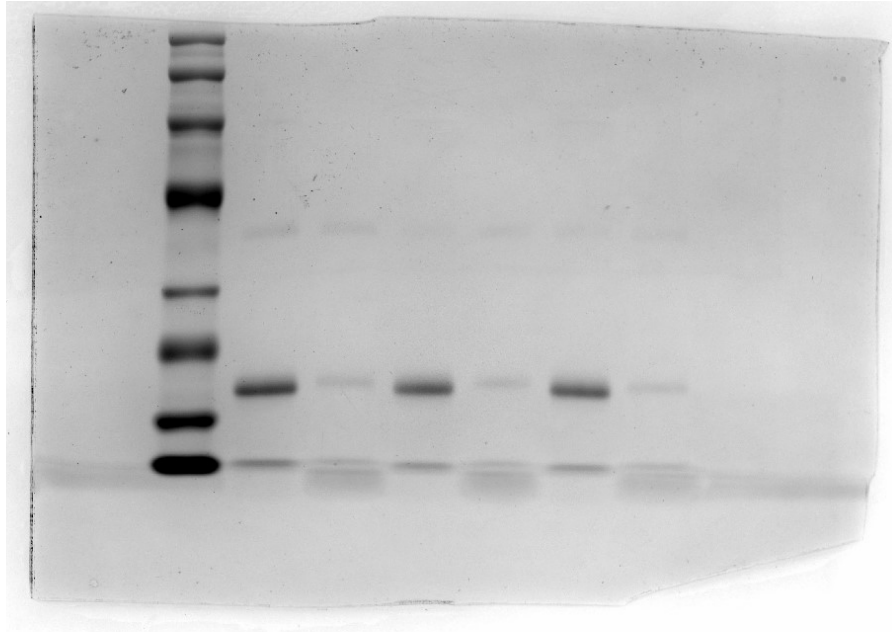

Fig. S2 SDS-PAGE results of uPA and tLipo@UA after Coomassie blue staining. The uncropped image of Fig. 1d.

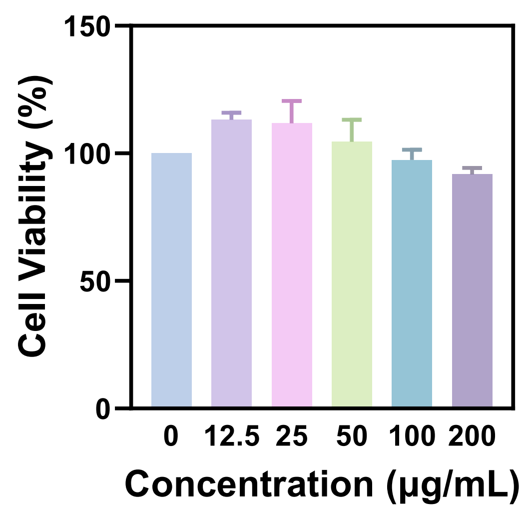

Fig. S3 MTT results of the treatment of tLipo@UA to bEnd.3 cells in 24 h.

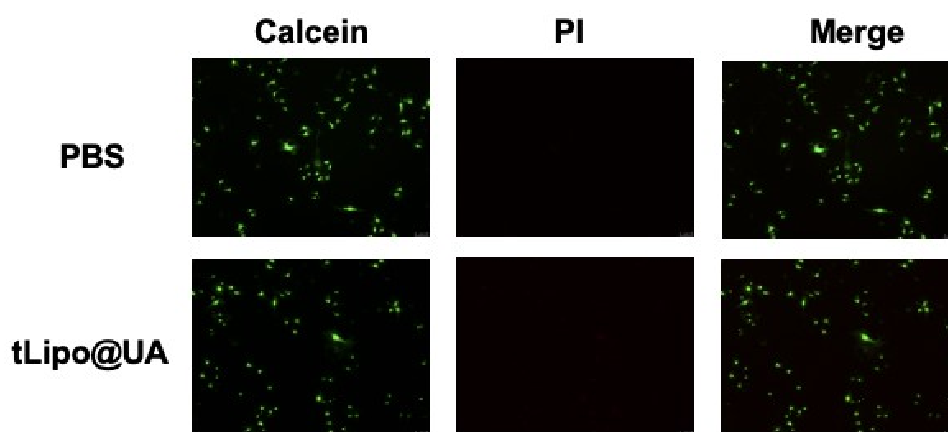

Fig. S4 Live/Dead cell staining results of the treatment of PBS or tLipo@UA to bEnd.3 cells in 24 h.

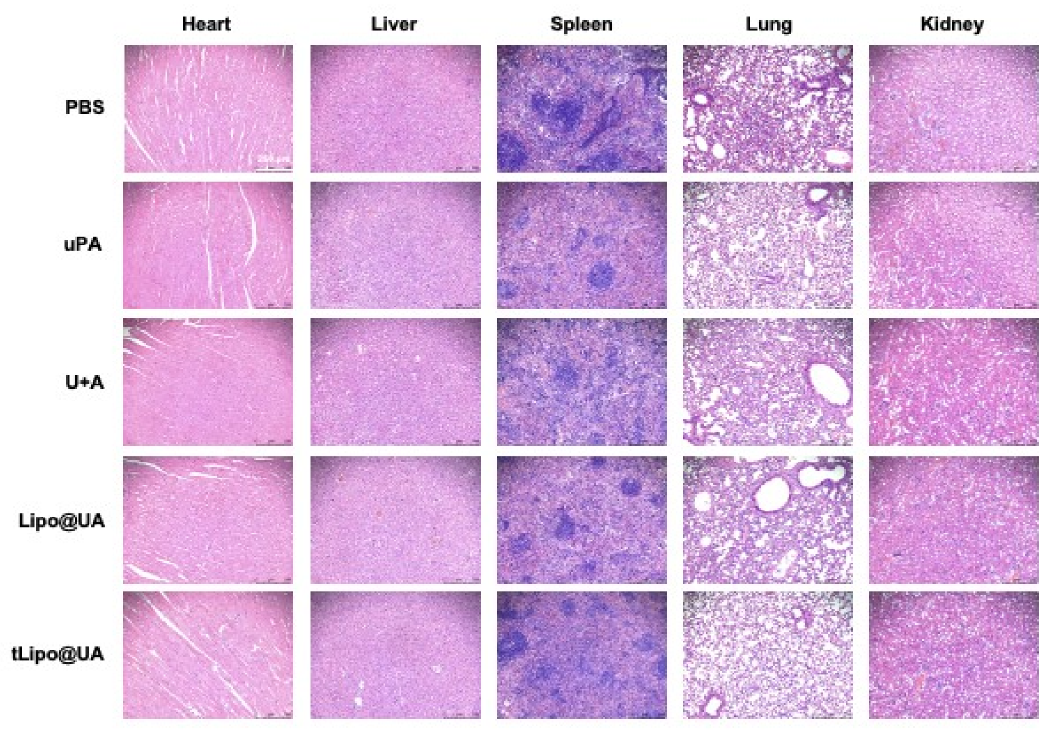

Fig. S5 H&E staining results of the treatment of PBS, uPA, U+A, Lipo@UA or tLipo@UA to KM mice after harvesting the heart, liver, spleen, lung, and kidney.

Supporting table

Tab. S1 Encapsulation efficacy of ASA and uPA.

	ASP	**uPA**
EE（%）	**83.71%**	**52.79%**