Supporting information

Recombinant supercharged polypeptides for safe and efficient heparin neutralization

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Materials

All chemicals were purchased from commercial suppliers and used without further purification. Heparin, methylene blue (MB), Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and polyethyleneimine (PEI) were ordered from Sangon Biotech. Two-stage kit, BiophenTM Anti-Xa (221005), was ordered from HYPHEN BioMed. Human blood from a healthy anonymous human donor.

SUPs preparation and characterization

The preparation of recombinant SUP proteins followed the reported protocol.[1] The obtained product was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Fig. S1).

Circular dichroism (CD)

CD spectra were recorded on a Chirascan Plus spectrophotometer (Applied Photophysics Ltd., England) under a nitrogen atmosphere. Experiments were performed in a quartz cell with a 1 cm path length. All SUPs were dissolved in PB or PBS at a concentration of 1 mg mL^{-1}.

MB displacement assay

A calibration curve was first plotted against heparin concentration with the help of MB in PB. Briefly, 50 µL of 10 µg mL^{-1} MB were mixed with 50 µL heparin with different concentrations (0, 0.6, 1.2, 1.8, 2.4 and 3.0 µg mL^{-1}) in PB, and the solutions were thoroughly mixed. The solutions were placed in a 96-well plate and read on a BioTek Cytation 3-microplate reader at room temperature. The absorption intensity ratios (A (664 nm)/A (568 nm)) against heparin concentrations were plotted as the calibration curve (Fig. S2a).

To determine the heparin-binding performance, different concentrations of binders in PB were mixed with heparin, yielding 100 µL PB solutions containing 3 µg mL^{-1} heparin and binders in desired mass ratios. Then, 50 µL of the solution was added to 50 µL MB solution (10 µg mL^{-1}) and the absorption spectra (450-750 nm) were recorded with a BioTek Cytation 3 microplate reader in a 96-well plate at room temperature. The absorbance intensity ratio A (664 nm) / A (568 nm) was used to determine the remaining heparin concentration in the solution and the heparin-absorbing ability was determined using the following equation:

$$\%\text{Neutralization} = [\,(c_0 - c_i)/c_0] \times 100$$

where c_0 is the derived heparin concentration at a mass ratio of 0 for all binders and c_i is the one at desired mass ratio. Measurements were performed using triplicate samples.

Anti-Xa assay

Heparin neutralization with binders was evaluated using a commercial two-stage kit, Biophen™ Anti-Xa (221005). Binders of different concentrations were added to heparin solution in PBS, giving a final heparin concentration of 0.04 IU mL^{-1} and desired mass ratios (binder/heparin). Kit reagents were utilized according to the manufacturer's instructions. To run the calorimetric assay, 40 µL of the binder-heparin solution was added to a 96-well microplate followed by the addition of 40 µL of antithrombin and incubation for 2 min. Then, 40 µL of factor Xa was added

and incubated for another 2 min. Afterward, 40 µL of the factor Xa-specific chromogenic substrate was added to the solution and let to react for 2 min. Finally, the reaction was quenched by introducing 80 µL of 20% acetic acid. The absorbance at 405 nm was recorded immediately using a BioTek Cytation 3-microplate reader. The anticoagulant activity is inversely proportional to the measured absorption intensity, and the percentage of neutralization was determined using a calibration curve constructed according to the manufacturer's instructions (Fig. S2c). Measurements were performed using triplicate samples.

Dynamic light scattering (DLS) measurement

The size determination was carried out on a Zetasizer Nano-ZS90 instrument (Malvern, UK) with a 4 mW He−Ne ion laser at the wavelength of 633 nm and an Avalanche photodiode detector at an angle of 173°. Zetasizer software (Malvern Instruments) was used to attain the data. Cumulant analysis was used to obtain the intensity mean value of the complex size, that is, the hydrodynamic diameter. Experiments were carried out at 25 °C. Heparin solutions were prepared by diluting 10 mg mL^{-1} heparin stock solution to 0.01 mg mL^{-1} in 0.3 mL buffer (PB or PBS). The heparin solutions were titrated with 2 µL of binder solutions resulting in total sample volumes of 20 µL. Measurements were carried out in PB with or without 150 mM NaCl. After every addition, the samples were allowed to equilibrate for 1 min. Each titration series was carried out three times, and all titration points were measured three times.

Quartz crystal microbalance with dissipation monitoring (QCM-D).

Interaction between surface immobilized SUPs or PS and heparin was investigated by using gold-coated sensors and a QCM-D unit (E4 instrument, Q-Sense AB, Sweden). The sensors were firstly cleaned with UV/ozone treatment for 15 min, followed by the immersion in a 0.1 wt % PEI for 30 min to absorb a PEI layer. Afterwards, the PEI coated sensors were thoroughly rinsed with Milli-Q water and dried with nitrogen gas. Heparin coverage was performed *in situ* to establish irreversible binding and full surface coverage before binder solution injection. After reaching a stable baseline with heparin solution, buffer solution was applied to rinse and remove loosely bound molecules. Finally, the binder solutions were applied, and the shifts in dissipation and frequency were monitored. All target molecules were dissolved in PB without or with 150 mM NaCl to yield a 0.1 mg mL^{-1} concentration. All solutions were filtered by using 0.45 µm filters prior to tests. Experiments were performed at a constant flow rate of 20 µL min^{-1}, and the temperature was maintained at 23 °C.

Haemolytic assay

The detailed procedure for the hemolysis assay has been previously reported.[2] Generally, fresh red blood cells from rats were centrifuged at 500 × g for 5 min and the plasma was gently removed. The remaining red blood cells were washed with 1× PBS three times and redispersed to the initial volume in 1× PBS. The red blood cells were diluted 25× and split into 96-well culture plates (100 µL/well). The concentrated sample solutions (100 µL) in 1× PBS were added to each well, resulting in the desired final compound concentrations (50−500 µg mL^{-1}). One hundred microliters of 2% Triton X-100 in 1× PBS and 100 µL of 1× PBS were added as positive and negative controls, respectively. After incubation at 37 °C for 1 h, the plates were centrifuged for 5 min at 500 × g to pellet intact erythrocytes, and 100 µL of the supernatant from each well was

delicately transferred into a clear 96-well plate. The resulting hemoglobin in the supernatant was measured at 540 nm with a microplate reader (Cytation 3, Biotek). The percentage of hemolysis was calculated as follows:

$$\%\text{Hemolysis} = [(A_{\text{sample}} - A_{\text{negative control}})/(A_{\text{positive control}} - A_{\text{negative control}})] \times 100$$

The measurements were performed using triplicate samples.

For the haemolytic assay of K72 with human blood, the same procedures were performed. The experiments involving human blood were performed in accordance with the guidelines of clinical research at Lishui Hospital, and experiments were approved by the ethics committee at Lishui Hospital Zhejiang University. Informed consents were obtained from human participants of this study.

CCK-8 assay

L929 cells were cultured in DMEM supplemented with 10% FBS. Cells were seeded at a density of 1×10^4 per well in 96-well plates. After overnight culture, cells were cultured with 50, 100, 200, 500 μg mL^{-1} of K9, K18, K36, K72, K144 and PS for 24 h. After incubation with binders, the solutions were removed and each well was washed with PBS buffer. 10 μL of CCK-8 solution mixed with culture medium (DMEM with 10% FBS) was added to each well. Afterwards, the plate was incubated for 2 h. Finally, the optical density of each sample was measured at 450 nm. For K72, three more cell lines (human umbilical vein endothelial cells (HUVEC), mouse skeletal muscle cells (C2C12) and human embryonic kidney 293 cells (293T)) were used to evaluate the cytocompatibility with the same concentrations for 24 h.

Animals

Sprague-Dawley (SD) rats with body weights of 250−300 g at 10−12 weeks of age (Life River, Beijing, China) were used for our rat model studies. Rats were maintained at an ambient temperature of 20 ± 2 °C on a 12:12 light-dark cycle environment with free access to water and food. All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Wenzhou Medical University and approved by the Animal Ethics Committee of Wenzhou Medical University (SYXK-2021-0020).

In vivo rat tail transection

The procedure followed a reported protocol with slight modification.[3] Briefly, twenty SD rats (male, 250−300 g) were randomly divided into four groups and anesthetized by standard anesthesia procedures. The rats were injected intravenously at the tail with saline, heparin (200 U kg^{-1}), or heparin with K72 or PS (5 mg kg^{-1}), respectively. After 10 min, the scalpel was cleaned and disinfected. Then each tail was transected with a scalpel at a site 20 mm from the end of tail. The blood was collected. The total bleeding time was measured until no visible blood flow was observed and the blood was weighted.

Blood samples were also collected from the ocular vein and centrifuged for various blood-index measurements through a biochemical autoanalyzer (type 7170, Hitachi, Japan). Meanwhile, the rats were sacrificed and their major organs, including heart, liver, spleen, lung, and kidney, were harvested and sectioned for H&E staining.

In vivo rat liver injury

The procedure followed a reported protocol.[3] Briefly, SD rats (male, 250−300 g) were divided into four groups of five, and anesthetized. Subsequently, rats were injected intravenously at the tail with saline, heparin (200 U kg^{-1}), or heparin with K72 or PS (5 mg kg^{-1}), respectively. After 10 minutes, rats were placed on their backs and the abdomen was opened exposing the liver. A wound with a length of 1 cm, 2 mm deep in the left lobe of the liver was cut by a razor blade. The time for complete arrest of bleeding (no blood flow within 10 s) was recorded, blood was collected and weighed with filter paper every 10 seconds until bleeding stopped.

Serum biochemical analysis.

Saline or K72 was intravenously administered into healthy rats at the tail (n = 5 for each group) and the body weight was monitored for 14 days. At 14 days post injection, blood samples were collected from the ocular vein and centrifuged for various blood-index measurements through a biochemical autoanalyzer (type 7170, Hitachi, Japan). Meanwhile, the rats were sacrificed and their major organs including heart, liver, spleen, lung, and kidney were harvested and sectioned for H&E staining.

The pharmacokinetics of K72

To obtain the pharmacokinetics of K72, K72 was fluorescently labeled with Cyanine5 NHS ester (5 equivalent) overnight. Then, the solution was thoroughly washed and concentrated via filtration with vivaspin tubes with a molecular cutoff of 10 kDa. Subsequently, rats were injected intravenously at the tail with the labeled K72 (5 mg kg^{-1}), and the blood was collected at desired time points (5 min, 10 min, 20 min, 30 min, 1 h, 2 h, 4 h, 6 h, 10 h, 24 h and 30 h). The collected blood was haemolyzed by diluting 20 time in Milli-Q water, and the fluorescence was recorded with an excitation of 650 nm. Emission peak at 570 nm was selected to calculate the relative concentration of K72 in blood. The fluorescence recorded at 5 min was assigned as the starting K72 concentration in blood and the remaining K72 in blood at different time points were calculated based on the following equation:

Relative K72 content in blood (%) = $F_x/F_{5\ min} \times 100$

In the equation, F_x indicates the fluorescence intensity (570 nm) at corresponding time points.

Statistical analysis.

All data are shown as mean ± standard deviation. Statistical values are indicated in figures according to the following scale: ** means $p < 0.005$ and *** means $p < 0.001$.

Supplementary figures

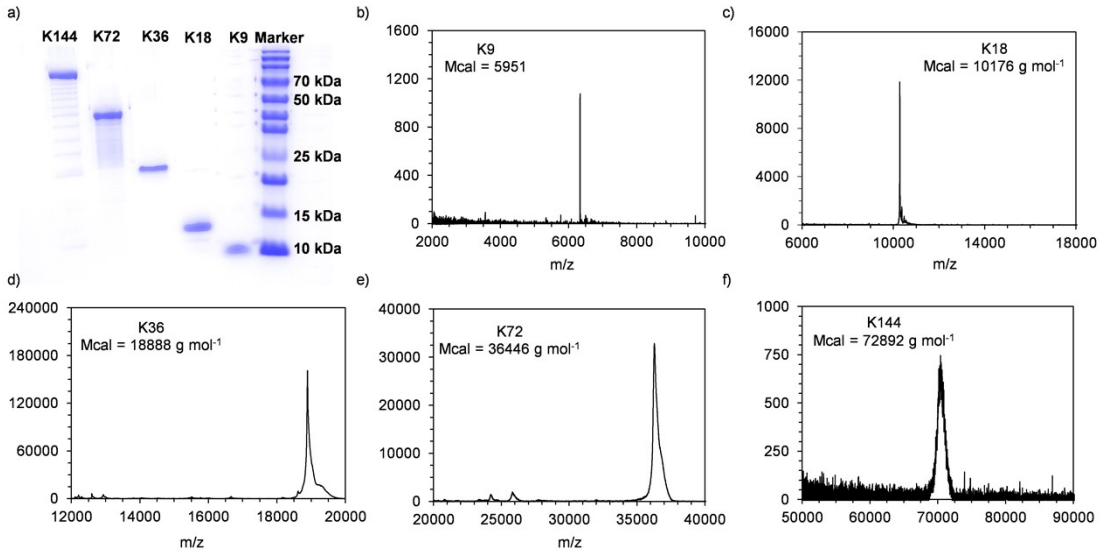

Fig. S1. Characterization of SUPs by (a) SDS-PAGE and (b-f) MALDI-TOF MS.

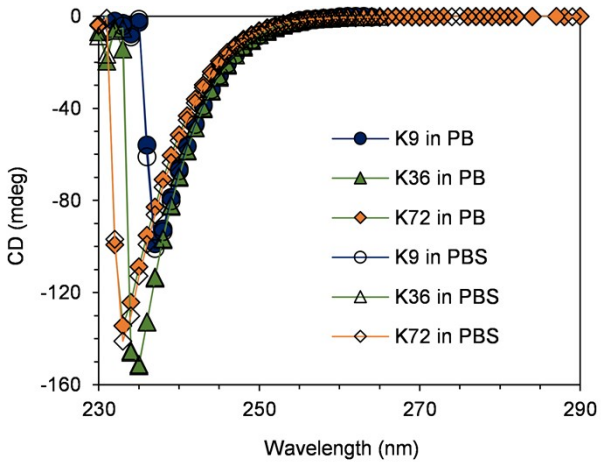

Fig. S2. CD spectra of K9, K36 and K72 in PB or PBS (1 mg mL^{-1}).

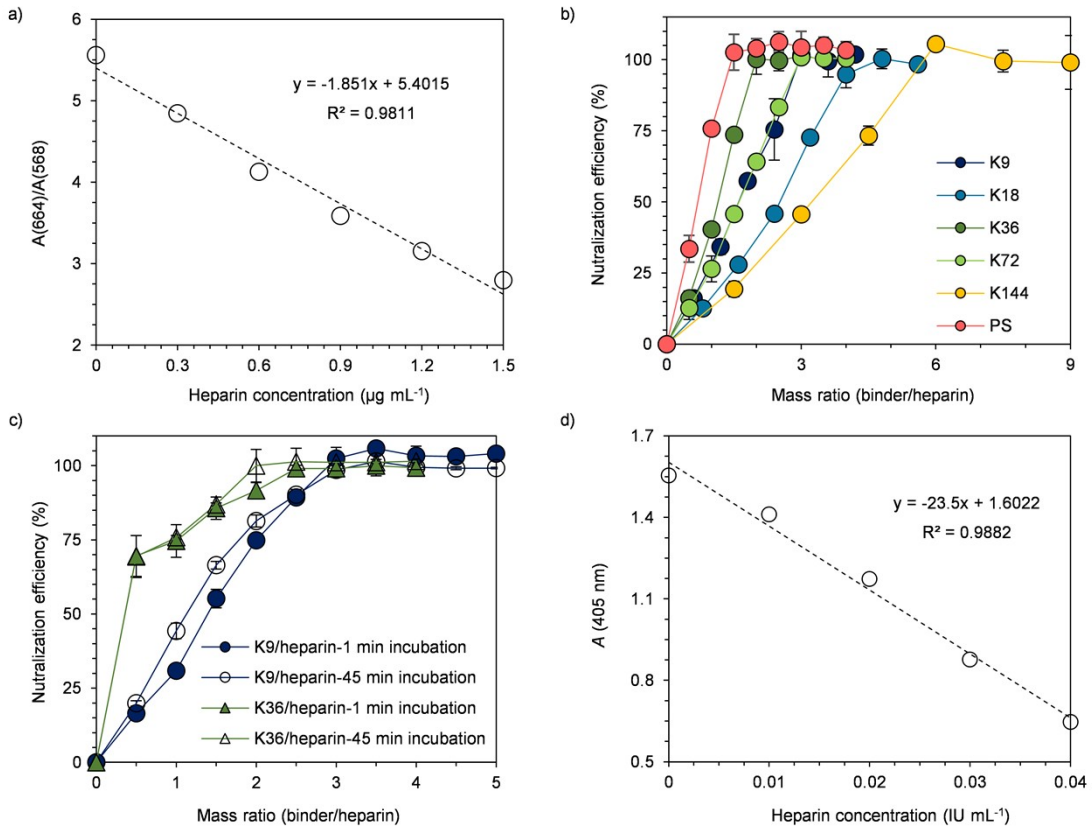

Fig. S3. (a) The calibration curve for MB-displacement assay; (b) The heparin-reversal ability of SUPs and PS measured with MB-displacement assay (heparin: 1.5 μg mL^{-1}); (c) Evaluation of the effect of incubation time on the binding performance with MB-displacement assay. Measurements were performed using triplicate samples, and the averaged results with standard deviation are presented; (d) The calibration curve for anti-Xa assay.

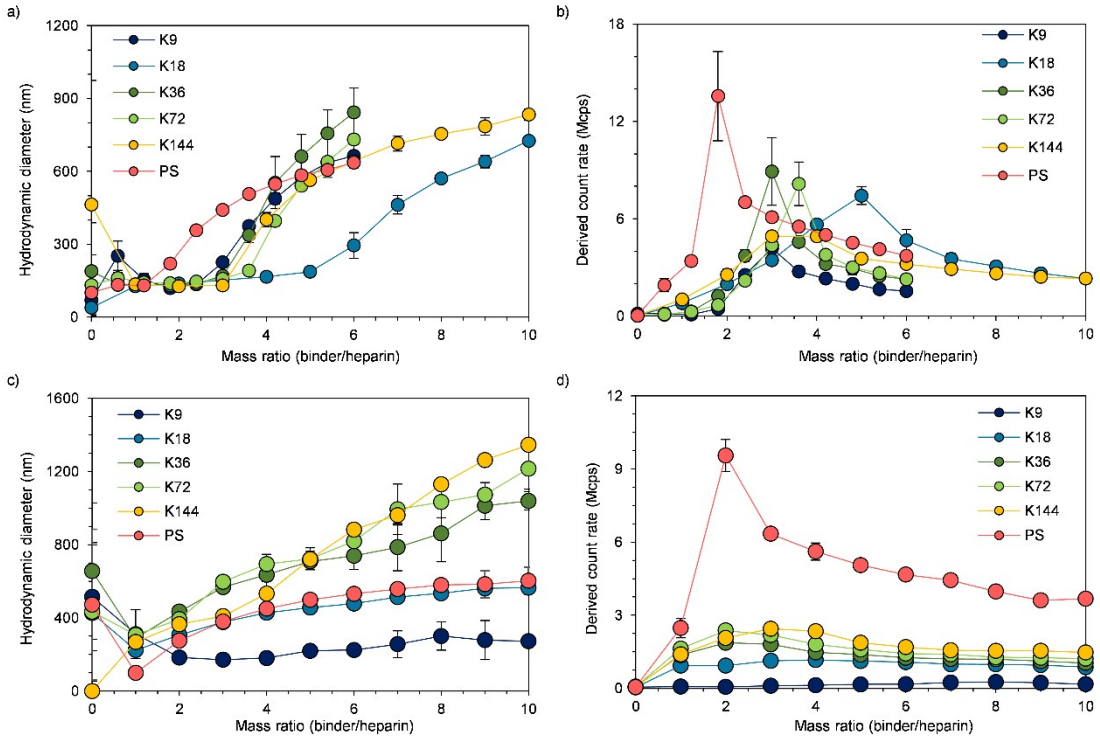

Fig. S4. Titration of heparin (0.01 mg mL^{-1}) with SUPs or PS in (a,b) PB and (c,d) PBS monitored with DLS. The hydrodynamic diameter (a,c) and derived count rate (b,d) were used as indicators for the successful complexation. Titrations were performed using triplicate samples, and the averaged results with standard deviation are presented.

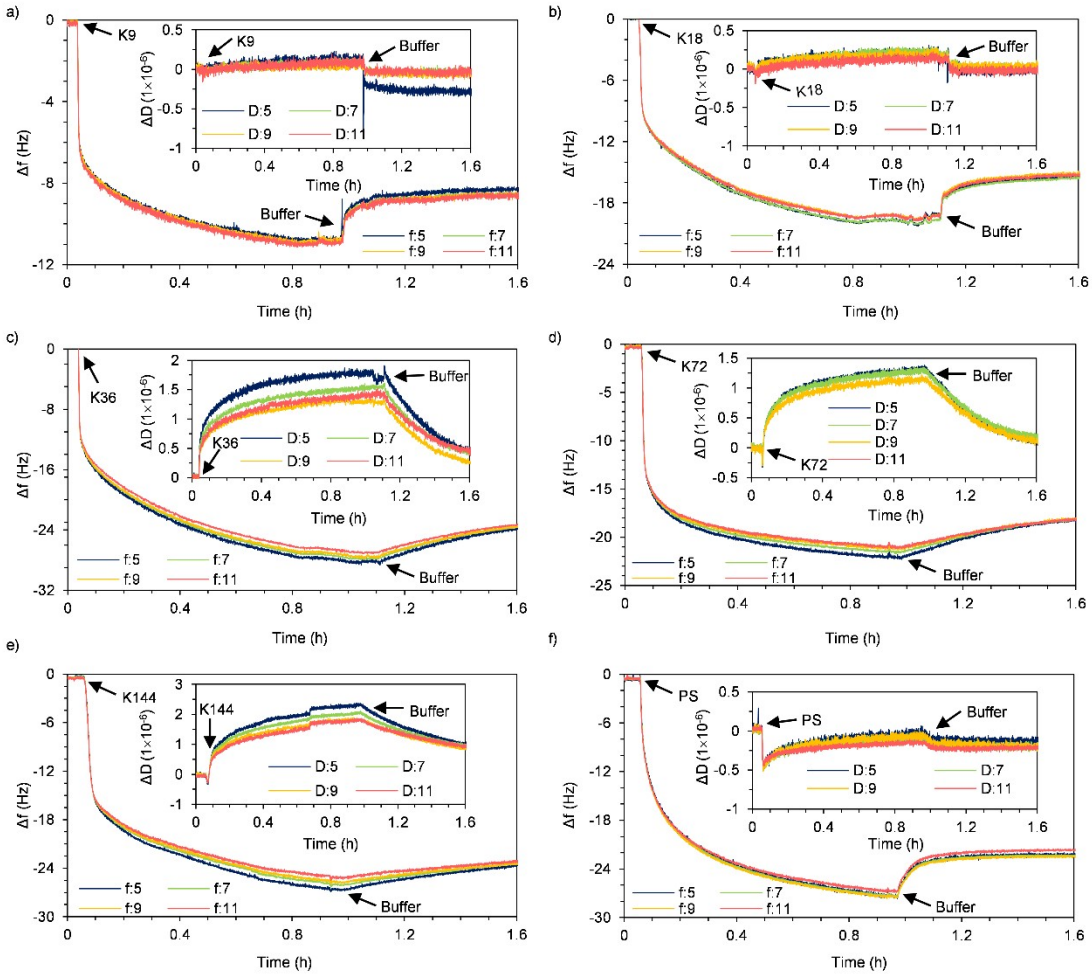

Fig. S5. Frequency shifts (*Δf*) and energy dissipation (*ΔD*) graphs (insets) from QCM-D measurements investigating the binding of (a) K9, (b) K18, (c) K36, (d) K72, (e) K144, and (f) PS to heparin in PB under continuous flow (binder concentration: 0.1 mg mL^{-1}).

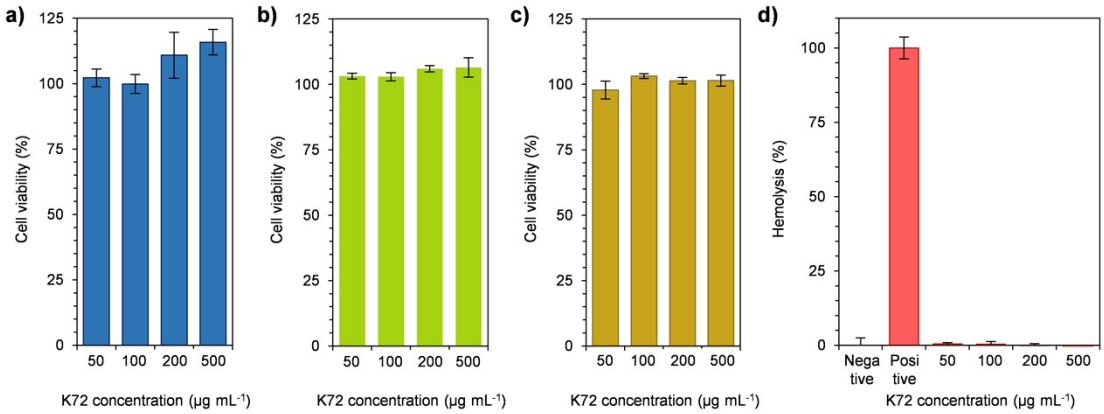

Fig. S6. Evaluation of the biocompatibility of K72 with (a) HUVEC, (b) C2C12, (c) 293T and (d) human RBCs.

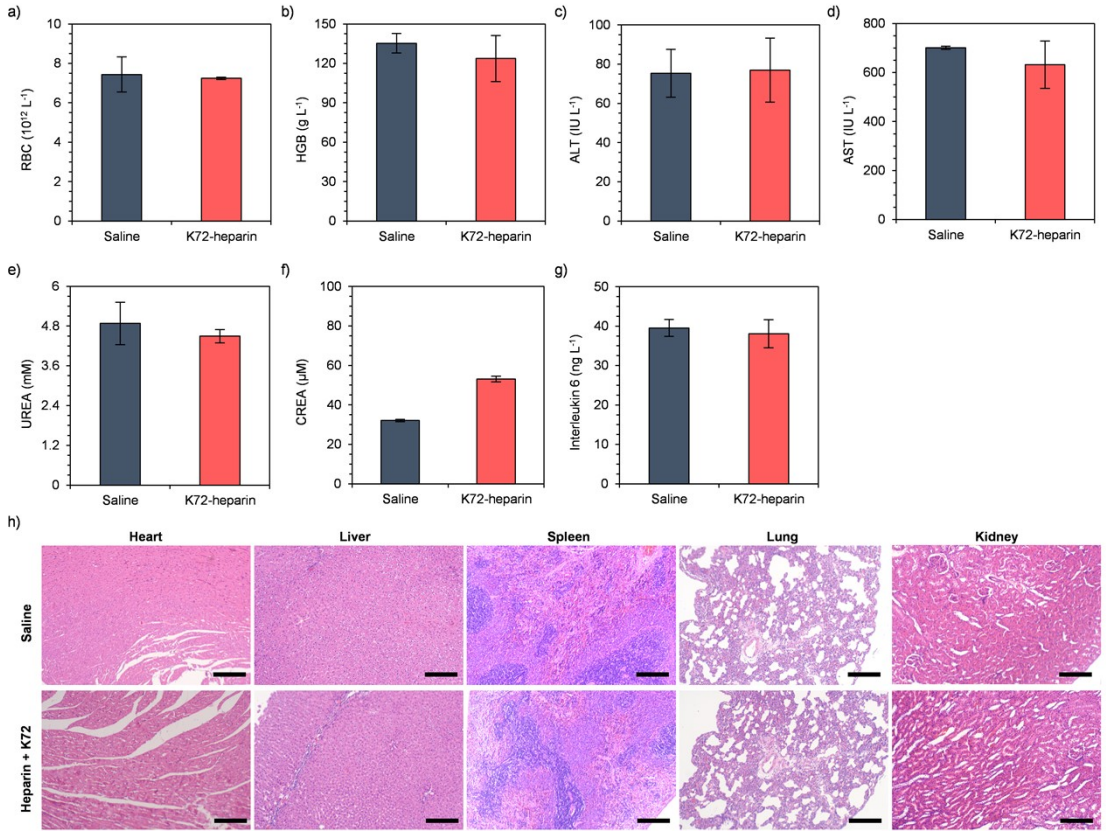

Fig. S7. Evaluation of the *in vivo* biocompatibility of heparin (200 U kg^{-1}) with successive K72 (5 mg kg^{-1}) administrated intravenously through the tail vein. (a-g) Comparison of typical blood routines and biochemical indexes. Data are presented with means ± standard error of the mean (S.E.M.) (*n* = 5); (h) H&E histopathologic examination of the major organs (from left to right: heart, liver, spleen, lung, and kidney). Scale bar: 200 μm.

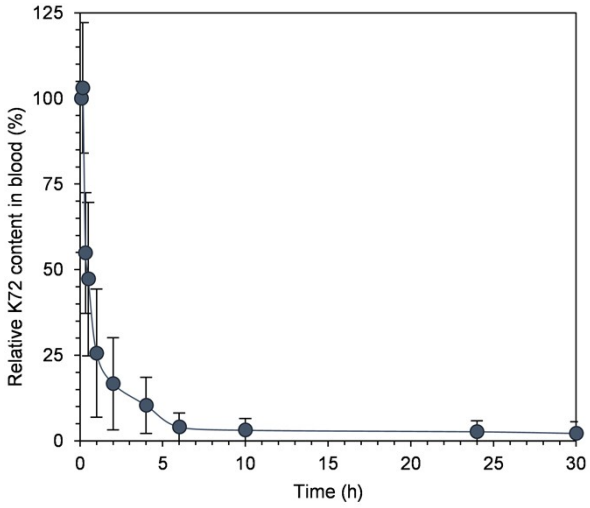

Fig. S8. The pharmacokinetics of K72 in rats. K72 concentration: 5 mg kg^{-1}. Data are presented with means ± standard error of the mean (S.E.M.) (*n* = 5).

References

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