Supporting Information

Ascidian-Inspired Heparin-mimetic Magnetic Nanoparticles with Potential for Application in Hemodialysis as Recycling Anticoagulant

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Figure S1-S5
MATERIALS.

FeCl$_2$.4H$_2$O, FeCl$_3$.6H$_2$O and sodium alginate (very low viscosity) (SA) were purchased from Alfa Aesar (China) Chemical Co. Ltd; Ammonia water, oleic acid, 3,4,5-trihydroxy-phenylalanine (TOPA), N, N'-Dicyclocarbodiimide (DCC), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Sigma (USA). Bovine serum albumin (BSA), bovine serum fibrinogen (BFG) were purchased from Sigma (USA). Phosphate-buffered saline (PBS, pH = 7.2-7.4) solution was a buffer solution. N, N-dimethylformamide (DMF) (Chemical Reagent Factory of Kelong, China) were dried by stirring with CaH$_2$ and then distilled before use. All the other chemicals (analytical grade) were obtained from Chemical Reagent Factory of Kelong (China), and were used without further purification. Dialysis membranes (MWCO = 8 000 and 14 000 Da) were obtained from Solarbio (Canada).

SYNTHESIS and CHARACTERIZATION

Synthesis of HLSA and MNP

The synthetic procedure of HLSA was shown as follows: 5 g of H$_2$SO$_4$ was added to 50 mL of DMF, and the mixed solution was allowed to cool to room temperature. Then, 5 g of SA was dissolved in the H$_2$SO$_4$/DMF solution, and then 10 g of DCC was added in the mixture. The reaction was carried out at room temperature for 2 h with continuous stirring. At the end of the reaction, the precipitate was removed by filtration using a glass sand funnel. Then, three volumes of dichloromethane were added to the supernatant to form a second precipitate. The precipitate was further dissolved into 100 mL of 0.5 M sodium hydroxide and the solution was kept at ambient temperature for about 30 min. The DCC-urea precipitate was then removed by filtration. The resulting solution containing the sodium alginate sulfate was dialyzed against deionized (DI)
water for 5 times, and lyophilized to obtain the final product.

The synthetic procedure of MNP was shown as follows: 1.8 g FeCl$_2$.4H$_2$O and 4.2 g FeCl$_3$.6H$_2$O were added to 100 mL of DI water. The mixed solution was introduced to a three-neck round-bottom flask equipped with mechanical agitator and was carried out at room temperature with vigorous stirring for about 0.5 h. After vacuuming and back-filling nitrogen atmosphere several times, the mixed solution was heated to 50 °C and kept for 0.5 h. 25 mL ammonia water was dropwise added into the flask and the flask was heated to 80 °C. Then, 2 mL oleic acid was dropwise added into the flask, and the reaction system at 80 °C was kept for 1 h. The reaction mixture was cooled to room temperature, and the residual reagents were removed by dispersing the resulting solution containing MNP into DI water and ethanol, followed by magnetic adsorption, and the purification procedure was repeated several times.

Synthesis of TMNP and HMNP

Typically, MNPs were well dispersed in PBS and quickly mixed with TOPA solution. Then the well dispersed solution was carried out (25 °C) with vigorous stirring for about 12 h in alkaline environment (adjust by 0.02 M NaOH). The residual impurity in the product were removed by dispersing the resulting solution containing TMNP into DI water, followed by centrifugation; and the purification procedure was repeated for several times to remove all the impurities.

HMNPs were prepared via a two-step reaction, and the method was described as follows. Briefly, 1 g of HLSA was dissolved in 30 mL MES buffer (0.05M, pH = 5.3, 0.1 M NaCl). After dissolving completely, EDC (0.3834 g, 2 mmol), NHS (0.1151 g, 1 mmol) and 1 g of TMNPs were added sequentially, and the reaction was carried out for 24 h with vigorously stirring under nitrogen protection at 25 °C, and the pH was maintained at 5.3 by the addition of 1 M HCl. Then, saturated NaCl solution and 60 mL
cold ethanol were added sequentially. After centrifugation at 1 000 g for 10 min, the precipitated HMNPs were re-dispersed in 1 M NaCl solution, and ethanol was added again. The volume ratio of ethanol to NaCl solution was 10:1. This purification step was repeated three times to minimize the electrostatic interaction between the HLSA and TOPA. At last, HMNPs were dialyzed against DI water using a dialysis membrane (MWCO: 14 000) for 1 d under acidified water (pH = 5, adjusted by 1 M HCl, to avoid pyrogallol oxidation).

**Characterization**

Fourier transform infrared (FT-IR) spectra were obtained on a Nicolet-5700 spectrophotometer (Nicol, US) between 4000 and 650 cm⁻¹. The morphology of HMNP was examined via a Tecnai G2 F20 S-TWIN instrument (TEM, FEI, USA). The crystal structure was determined using an X'Pert PRO instrument (PANalytical, NL) with CuKα radiation. The relative amount of the magnetic nanoparticles associated with the TOPA and HLSA were determined using thermogravimetric analysis (TGA, TA instruments Q50, USA). Samples were dried in a vacuum oven for 48 h and analyzed in the platinum plate at a heating rate of 10 °C/min from 25 to 500 °C under nitrogen atmosphere. The thermal property of HMNP was measured using a differential scanning calorimeter (DSC, TA instruments DSC250, US) at a heating rate of 10 °C/min from 25 to 500 °C under nitrogen atmosphere. The weight of the samples taken for each record was 6-9 mg. The average degrees of substitution (DS) per monosaccharide in HLSA were measured and calculated by elemental analysis (EA). The number-average molecular weight of the HLSA was determined by gel permeation chromatography (GPC) with a Waters 1515 device (USA), in which two PL gel columns (10 μm, 104 Å; 10 μm, 500 Å) were used, monodisperse polystyrene was the standard, and the mobile phase was dimethylformamide. The sample concentration was
1.0 g/L. The detector was refractive index detector, and the flow rate was 1.0 ml/min.

**BLOOD COMPATIBILITY**

The collection of human blood, preparation of plasma and related experiments were all performed in the Institute of Blood Transfusion. All the experiments were performed in compliance with the relevant laws and institutional guidelines, and all the experiments were informed and approved by the institutional committee and performed by the staff of the institute.

**Protein Adsorption:** Protein adsorption experiments were carried out using BSA and BFG solutions under static conditions. First, 1 mL nanoparticle/phosphate buffer solution (PBS, pH = 7.4) with a concentration of 250 mg/mL was added into PBS, containing BSA or BFG with a concentration of 5 mg/mL, and incubated at 37 °C for 1 h; it was subsequently rinsed slightly with PBS and DI water. Then the nanoparticles were collected by using a magnet. The protein concentration in supernatant was determined by the Micro BCA™ Protein Assay Reagent Kit (PIERCE), and the adsorption rates of BSA and BFG were calculated. The experiments were repeated at least three times, and the data were expressed as mean ± SD.

**Clotting Time:** To evaluate the anticoagulant performance of MNP, TMNP and HMNP, activated partial thromboplastin time (APTT) and thromboplastin time (TT) were measured by a semi-automatic blood coagulation analyzer CA-50 (Sysmex Corporation, Kobe, Japan).

The APTT test method was performed as follows. The whole blood was collected with a net venipuncture without tourniquet using vacuum tubes containing sodium citrate as anticoagulant (anticoagulant to blood ratio, 1:9) and centrifuged at 4000 rpm for 15 min to obtain platelet-poor plasma (PPP). Then, 150 mg nanoparticles were dispersed into 5 mL PBS (pH = 7.4) for 1 h. Then, 5 μL nanoparticle/PBS was added
to 0.1 mL of fresh PPP after even shaking. After incubating at 37 °C for 30 min, 50 μL of APTT agent (Dade Actin Activated Cephaloplastin Reagent, Siemens; incubated 10 min before use) was added, followed by the addition of 50 μL of the incubated PPP, and incubated at 37 °C for 3 min. Thereafter, 50 μL of 0.025 M CaCl₂ solution was added, and then the APTT was measured. For TT test, 50 μL incubated PPP was mixed well with 100 μL of TT agent (Thromborel® S, Siemens; incubated 10 min before use) at 37 °C for 2 min, and the TT was measured. At least three measurements were averaged and the results were analyzed by a statistical method.

**Anticoagulant Activity:** In order to study the anticoagulant activity of HMNP, the APTT and TT of HMNP with different concentrations were measured using a semi-automatic blood coagulation analyzer CA-50 (Sysmex Corporation, Kobe, Japan). At least three measurements were averaged and the data were analyzed by a statistical method.

The measured samples were prepared as follows. The HMNP dispersion was prepared by dispersing 150 mg HMNP in 5 mL PBS. High-speed shaking into uniform dispersion, confirmed the phenomenon of non-caking or aggregate after measuring. The concentrations of the measured samples were 25, 50, 100, 150 μg per 100 μL plasma, respectively. At least three measurements were averaged and the data were analyzed by a statistical method.

**Hemolysis Test:** For hemolysis test, the red blood cells (RBCs) were incubated with MNP, TMNP, and HMNP at 37 °C for 3 h, respectively. DI water was incubated with RBCs to prepare control sample. The absorbance of the released hemoglobin was measured by an UV-vis spectrometer (UV-1750, Shimadzu Co., Ltd, Japan) at 540 nm, after the incubated solutions were centrifuged at 10016 g for 3 min. And then the hemolysis ratio could be calculated by eq. (S1):
Hemolysis ratio = \frac{Suspensions_{\text{abs}} - \text{Negative control}_{\text{abs}}}{\text{Positive control}_{\text{abs}} - \text{Negative control}_{\text{abs}}} \times 100\% 

**Elisa Kit Method:** For platelet activation test: The whole blood was collected with a net venipuncture without tourniquet using vacuum tubes containing sodium heparin as anticoagulant (anticoagulant to blood ratio, 1:9) and centrifuged for 15 min at 2500 g (4 °C) to obtain platelet-rich plasma (PRP). The plasma was diluted 10 fold in the PF4 Sample Diluent to obtain PF4 Standard Solution. A series of graded PF4 Standard Solution/PF4 Sample Diluent (1/0, 0.5/0.5, 0.2/0.8, 0.1/0.9, 0.05/0.95, 0/1) were generated by reconstituting the Standard Solution with Sample Diluent, and the Sample Diluent served as the zero standard. Then the plasma was mixed with specific inhibitors. The control experiment was conducted simultaneously using the same method without adding samples. 200 μL of PF4 Standard Solution was added per well, and the wells were covered with a sealing tape and incubated for 1 hour. After incubating, the plate was washed as described above. Then, 200 μL of Conjugate Solution which was prepared by reconstituting Conjugate with 7.5 mL of Conjugate Diluent was added per well and incubated at room temperature for 1 hour. After incubating, the plate was washed as described above. Then, 200 μL of TMB Substrate was added to each well and incubated for 5 min. Finally, 50 μL of Stop Solution was added to each well, and the color would change. The whole experiment was manipulated free from light. The absorbance could be read on a microplate reader (the microplate reader was turned on and the program was set up in advance) at a wavelength of 450 nm immediately, and then the PF4 amount was calculated. At least three measurements were averaged to get a reliable value, and the results were analyzed by a statistical method.

For thrombin-antithrombin complex activation test: For coagulation activation test, the whole blood was collected using one-tenth volume of 0.1 M sodium citrate as an anticoagulant, and centrifuged for 10 min at 3000 g (4 °C) to obtain plasma. The TAT
reagents were freshly diluted and brought to room temperature before use. 120 ng/mL standard solution was generated by reconstituting the 180 ng of Human TAT Complex Standard with 1.5 mL of MIX Diluent. Prepared duplicate standard points by serially diluting the standard solution 1:3 with MIX Diluent to produce 40, 13.33, 4.444 and 1.481 ng/mL solutions, and the MIX Diluent served as the zero standard. Then the plasma was mixed with specific inhibitors. The control experiment was conducted simultaneously using the same method without adding samples. 50 μL of Human TAT Complex Standard was added per well, and the wells were covered with a sealing tape and incubated for 2 h (37 °C). Then the wells were washed for 7 times with 200 μL of Wash Buffer manually (Invert the plate each time and decant the contents hit 10-15 times on absorbent material to completely remove the liquid. 50 μL of Biotinylated Human Thrombin Antibody was added to each well and incubated for 1 hour. After incubating, the plate was washed as described above. Then 50 μL of Streptavidin-Peroxidase Conjugate was added to each well and incubated for about 30 min. The plate was washed as described above. Then 50 μL of Chromogrn Substrate was added per well and incubated for about 20 min or till the optimal color density developed. Gently tap the plate to ensure thorough mixing and break the bubbles in the well with pipette tip. Finally, 50 μL of Stop Solution was added to each well, and the color would change from blue to yellow. The whole experiment was manipulated free from light. The absorbance could be read on a microplate reader (the microplate reader was turned on and the program was set up in advance) at a wavelength of 450 nm immediately, and then the TAT amount was calculated. All the experiments were repeated three times, and the results were analyzed by a statistical method.

For Blood Related Complement Activation tests: For C3a test, the whole blood was collected using vacuum tubes containing disodium EDTA as anticoagulant
(anticoagulant to blood ratio, 1:9) and centrifuged for 15 min at 1000 g (4 °C) to obtain plasma. The plasma was diluted 500 fold in the C3a Sample Diluent to obtain Standard Solution. 5 ng/mL standard solution was generated by reconstituting the 9.6 ng of C3a Standard with 1.92 mL of Standard Diluent. Prepared duplicate standard points by serially diluting the standard solution 1:2 with Standard Diluent to produce 2.5, 1.25, 0.63, 0.31, 0.16 and 0.08 ng/mL solutions, and the C3a Standard Diluent served as the zero standard. Then the plasma was mixed with specific inhibitors. The control experiment was conducted simultaneously using the same method without adding samples. 100 μL of C3a Standard Solution was added to each well, followed by the addition of 50 μL of ELISA Diluent, and then the wells were covered with a sealing tape and incubated at room temperature for 2 h. After incubating, the plate was washed as described above. Then, 100 μL of Working Detector which was prepared by reconstituting 48 μL of Enzyme Concentrate with 120 mL of Detection Antibody was added per well and incubated for 1 hour. After incubating, the plate was washed as described above. Then, 100 μL of TMB One Step Substrate Reagent was added to each well and incubated for 30 min. Finally, 50 μL of Stop Solution was added to each well, and the color would change. The whole experiment was manipulated free from light. The absorbance could be read on a microplate reader (the microplate reader was turned on and the program was set up in advance) at a wavelength of 450 nm immediately, and then the C3a amount was calculated. All the experiments were repeated three times, and the results were analyzed by a statistical method.

For C5a test, the whole blood was collected using vacuum tubes containing disodium EDTA as anticoagulant (anticoagulant to blood ratio, 1:9) and centrifuged for 15 min at 1000 g (4 °C) to obtain plasma. The plasma was diluted 10 fold in the C5a Sample Diluent to obtain Standard Solution. 5 ng/mL standard solution was generated by
reconstituting the 9.6 ng of C5a Standard with 1.92 mL of Standard Diluent. Prepared
duplicate standard points by serially diluting the standard solution 1:2 with Standard
Diluent to produce 2.5, 1.25, 0.63, 0.31, 0.16 and 0.08 ng/mL solutions, and the C5a
Standard Diluent served as the zero standard. Then the plasma was mixed with specific
inhibitors. The control experiment was conducted simultaneously using the same
method without adding samples. 100 μL of C5a Standard Solution was added to each
well, followed by the addition of 50 μL of ELISA Diluent, and then the wells were
covered with a sealing tape and incubated at room temperature for 2 h. After incubating,
the plate was washed as described above. Then, 100 μL of Working Detector which
was prepared by reconstituting 48 μL of Enzyme Concentrate with 120 mL of Detection
Antibody was added per well and incubated for 1 hour. After incubating, the plate was
washed as described above. Then, 100 μL of TMB One Step Substrate Reagent was
added to each well and incubated for 30 min. Finally, 50 μL of Stop Solution was added
to each well, and the color would change. The whole experiment was manipulated free
from light. The absorbance could be read on a microplate reader (the microplate reader
was turned on and the program was set up in advance) at a wavelength of 450 nm
immediately, and then the C5a amount was calculated. All the experiments were
repeated three times, and the results were analyzed by a statistical method.

**CYTOTOXICITY**

**Human umbilical vein endothelial cells (HUVECs) culture:** $2 \times 10^4$ HUVECs
were grown in R1640 medium supplemented with 10% fetal bovine serum (FBS,
Hyclone, USA), 2 mM L-glutamine and 1% (V/V) antibiotics mixture (10000 U
penicillins and 10 mg streptomycin). Cultures were maintained in humidified
atmosphere of 5% CO$_2$ at 37 °C (Queue Incubator, Paris, France). Confluent cells were
detached from the culture flask with sterile PBS and 0.05% trypsin/EDTA solutions,
and the culture medium was changed every day.

**MTT Assay:** Different concentrations of TMNP and HMNP were co-cultured with HUVECs (approximately $2 \times 10^4$ cells/well), respectively. For the MTT assay, the viability of the cells was determined after cell culture for 3 d. After predetermined time intervals, 45 μL MTT solution (1 mg/mL in the test medium) was added to each well and incubated for 4 h at 37 °C. Mitochondrial dehydrogenases of viable cells selectively cleave the tetrazolium ring, yielding blue/purple formazan crystals. Then, 400 μL of ethanol was added to dissolve the formazan crystals. Thus, the quantity of the formazan dissolved in the ethanol reflected the level of cell metabolism. The solution was shaken homogeneously for about 15 min. The sample solution was aspirated into microtiter plates and the optical density was read in a Microplate reader (model 550, Bio-Rad, 492 nm). All the experiments were repeated three times and the results were expressed as means ± SD.

**RESULTS and DISCUSSION**

![Figure S1. XRD pattern for HMNP.](image)

**Figure S1.** XRD pattern for HMNP.
**Figure S2.** APTT for heparin and HLSA. (Values are expressed as mean ± SD, n = 3. *P < 0.05 compared with the values for the control sample, #P < 0.05 for the same sample at a concentration of, 1, 2.5, 5, 10 and 25 μg per 100 μL, respectively.)

**Figure S3.** Hemolytic activity of samples. (Values are expressed as mean ± SD, n = 3.)

Figure S3 shows the hemolysis ratios of human RBCs exposed with different samples for 3 h. As shown in the figure, TMNP and HMNP exhibited reduced hemolysis, since the directly interaction between MNP and RBC was avoided. Apparently, increasing the concentration of HMNP did not play a significant role in RBC rupture, at least within the tested concentrations. The results indicated that the HMNP exhibited good blood cell compatibility with very low hemolysis ratios below 0.5%, much lower than
the ISO standard (5%), which enables HMNP promising potential to satisfy clinical requirement.

**Figure S4.** MTT tetrazolium assays of HUVECs treated with different concentrations of TMNP and HMNP for 3 d. (Values are expressed as mean ± SD, n = 3.)

The cytotoxicity of TMNP and HMNP was examined by MTT assay. As shown in Figure S4, the viability of the HUVEC was not significantly affected (< 90%) till the concentrations of TMNP and HMNP were up to 5 μg/mL, indicating excellent cytocompatibility of TMNP and HMNP. Moreover, it was noticed that HMNP showed lower cytotoxicity than TMNP, implying that HMNP was good candidate for recycling anticoagulant.
Figure S5. Pump operation time and recycled percentage. (Values are expressed as mean ± SD, n = 3.)