Supporting Information

Hemocompatible nucleosome-inspired heparin-mimicking hydrogel microspheres for safe and efficient extracorporeal removal of circulating histones in critically ill patients

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1. EXPERIMENTAL SECTION

1.1. Materials

Polyethersulfone (PES) was purchased from BASF Chemical Company (Ultrason E6020P, Germany). N-vinyl-2-pyrrolidone (VP, 99.0%), acrylic acid (AA, 99.0%), 2-acrylamido-2-methyl-1-prroanesulfonic acid (AMPS, 98%), N, N′-methylene bis(acrylamide) (MBA, $C_7H_{10}N_2O_2$, for molecular biology, ≥ 99.0%), 2,2-azobisisobutyronitrile (AIBN, 98.0%), ammonium persulfate (APS, $(NH_4)_2S_2O_8$, AR, ≥ 98.0%), sodium dodecyl sulfate (SDS, 98.0%), sodium hydroxide (NaOH, 97.0%), heparin sodium salt (≥180 USP units/mg), ethanol (CH_3CH_2OH, AR, ≥ 99.7%), and phosphate-buffered saline (PBS, pH = 7.4) were purchased from Aladdin Reagent Co. Ltd. (Shanghai, China). N, N'-Dimethylacetamide (DMAc, ≥99.8%) was purchased from Chengdu Kelong Chemical Reagent Co. Ltd. (China). Bovine serum albumin (BSA) and hirudin were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). The ungraded mixture of calf thymus histones was purchased from Sangon Biotechnology Inc. (Shanghai, China). Micro BCA™ protein assay reagent kit was purchased from PIERCE Inc. Human complement fragment 3a (C3a) kit, and a human complement fragment 5a (C5a) kit were purchased from Cusabio Biotech, China. The deionized (DI) water used in all the experiments was prepared by a deionized lab water system. All the chemicals were used as received.

1.2. Preparation of the RCHMs

Table S1 Composition of the reaction solution

The reaction solution	AA		AMPS		MBA	APS	DI water
	(g)	(mol)	(g)	(mol)	(g)	(g)	(g)
A_1M_3	3.75	0.052	11.25	0.055	0.6	0.15	34
A_2M_2	7.50	0.104	7.50	0.036	0.6	0.15	34
A_3M_1	11.25	0.156	3.75	0.018	0.6	0.15	34
A_0M_0	/	/	/	/	/	/	50

1.3. Characterization of the RCHMs

1.3.1. Fourier transform infrared spectroscopy (FTIR)

The microspheres were freeze-dried for 24 h to remove moisture, and then the dried microspheres were placed in a mold and the samples were detected in the transmission mode of an FTIR spectrometer (Thermo Fisher Nicolet iS50, USA) with a scan range of 4000-500 cm^{-1} and a resolution of 2 cm^{-1}. The average of 32 scans was taken as the result for each sample tested.

1.3.2. Thermogravimetric analysis (TGA)

The microspheres were freeze-dried, and then the samples with a dry weight of about 5~10 mg was placed in a ceramic crucible. The TGA curves were obtained using a thermogravimetric analyzer (METTLER TOLEDO TGA/DSC 3+, Switzerland). The heating program was set to 50 °C constant temperature equilibrium for 30 min, followed

by heating from 50 °C to 800 °C at a heating rate of 10 °C/min, the whole test was carried out under N_2 atmosphere.

1.3.3. Elemental analysis (EA)

The microspheres were freeze-dried, and then the samples with a dry weight of about 2 mg were placed into an elemental analyzer (Elementar UNICUBE, DEU) to analyze the contents of carbon, nitrogen, sulfur, and hydrogen in different microspheres directly. Two parallel analyses were conducted for each sample and the final elemental results were averaged over the two analyses.

1.3.4. X-ray photoelectron spectroscopy (XPS)

The microspheres were freeze-dried and analyzed using an X-ray photoelectron spectrometer (Thermo Scientific ESCALAB Xi+, USA). The vacuum degree of the analysis chamber was 1×10^{-9} Pa, and the excitation source was an Al kα ray (hν = 1486.6 eV). The operating voltage was 13.4 kV, the filament current was 6 mA, and the signal accumulation was performed for five cycles. The resulting data were analyzed using the Thermo Scientific™ Avantage software.

1.3.5. Mercury intrusion porosimetry (MIP)

The microspheres were freeze-dried, and then the samples with a dry weight of approximately 200 mg were subjected to analysis in an Automatic Mercury Pressure Meter (MicroActive AutoPore V 9600, USA) to determine the specific surface area, porosity, and pore distribution of the microspheres.

1.3.6. Scanning electron microscopy (SEM)

The cross-sectional samples were rapidly frozen with liquid nitrogen and then sectioned with a blade. Subsequently, the samples were freeze-dried and sprayed with gold on the surfaces by ion sputtering (vacuum degree 8 kPa, current 6-8 mA). This was followed by observation under a scanning electron microscope (Phenom Pure, Netherlands).

1.4. Hemocompatibility evaluation

1.4.1. Blood collection

Fresh human blood was obtained from healthy volunteers (22−28 years old) and collected using 5 mL vacuum tubes (Jiangsu Kangjian Medical Products Co., Ltd.) for *in vitro* hemocompatibility evaluation assays. All hemocompatibility experiments complied with national guidelines (GB/T 16886.4-2003/ISO 10993-4:2002) and were approved and executed by West China Hospital of Sichuan University (ethical approval number: 2024553).

1.4.2. Blood cell counts

Blood cell count assay were performed to investigate the impact of the RCHMs on blood cells. The microspheres were previously immersed in PBS overnight. 100 mg of microspheres were added to 500 μL of fresh whole blood (EDTA anticoagulated, anticoagulant-to-blood ratio = 1:9 (v/v)) and incubated with the whole blood at 37 ° C for 30 min. The remaining blood was then collected. The whole blood cell counts were measured by an automated hematology cell analyzer (Mindray BC-5100, China) according to the provided instructions. The results were expressed as the mean ± SD (n = 3).

1.4.3. Hemolysis ratio

The measurement of hemolysis ratio was used to evaluate the erythrocyte compatibility of the microspheres. The microspheres were previously immersed in PBS overnight. 5 mL of whole blood (sodium citrate anticoagulated, anticoagulant-to-blood ratio = 1:9 (v/v)) was firstly added to 10 mL of calcium- and magnesium-free saline, and then centrifuged at 2000 rpm for 10 min to separate the erythrocytes from the plasma. This process was repeated at least five times. The erythrocytes obtained after centrifugation were diluted 20 times with saline to obtain red blood cell suspension. During the hemolysis test, 10 mg of microspheres were added into 1 mL of the diluted red blood cell suspension and incubated at 37 °C for 3 h. The negative control was prepared by combining 0.2 mL of red blood cell suspension with 0.8 mL of saline, while the positive control was prepared by combining 0.2 mL of red blood cell suspension with 0.8 mL of DI water. After centrifuging at 7000 rpm for 3 min, the suspensions were collected to measure the absorbance of the released hemoglobin at 540 nm by a UV-Vis spectrometer (UV-1750, Shimadzu Co., Ltd, Japan). The hemolysis ratios of the microspheres were then calculated using Equation S1

$$\text{Hemolysis ratio (\%)} = \frac{A_s - A_n}{A_p - A_n} \times 100\% \quad \text{(S1)}$$

where A_s, $A_{p,}$ and A_n are the absorbance of the samples, positive control, and negative control, respectively. The results were expressed as mean ± SD (n = 3).

1.4.4. Evaluation of complement activation

Serum concentrations of C3a and C5a were measured by commercial ELISA kits (Thermo Fisher Human Complement C3a ELISA Kit and Thermo Fisher Human

Complement C5a ELISA Kit) to evaluate complement activation. The microspheres were previously immersed in PBS overnight. 50 mg of microspheres in each group were incubated with 150 μL of fresh blood (anticoagulated with 16000 ATU/mL recombinant hirudin, anticoagulant-to-blood ratio = 1:40 (v/v)) at 37 °C for 30 min. The blood co-cultured with the microspheres was centrifuged at 6500 rpm for 10 min, and the supernatant was diluted (1/1000 for the C3a test, 1/25 for the C5a test). 100 μL of the diluted sample or standard solution was added to the ELISA test well plate coated with the specific antibody. The detections were conducted according to the respective instructions and the whole blood sample was employed as the control. The results were expressed as mean ± SD (n = 3).

1.5. *In vitro* histone adsorption

1.5.1. Static histone adsorption

To assess the adsorption performance of the RCHMs for histones, mixed calf thymus histones were added to PBS buffer to prepare a series of histone solutions with concentrations of 100, 200, and 300 μg/mL. Then, 200 mg of the RCHMs was added to 1 mL of histone solution and shaken at 37 °C for 4 h. After that, the histone concentration was measured by the Micro BCA™ protein assay reagent kit, and the removal ratio and adsorption capacity of histones by the RCHMs were calculated according to Equations S2 and S3, respectively.

$$\text{Removal ratio (\%)} = \frac{C_0 - C_t}{C_0} \times 100\% \quad \text{(S2)}$$

$$q = \frac{(C_0 - C_t)V}{m} \times 100\% \quad \text{(S3)}$$

where C_0 and C_t (μg/mL) are the initial and residual histone concentrations at time t, q (μg/mg) is the adsorption capacity of histone, V (mL) is the histone solution volume, and m (mg) is the wet weight of microspheres. The results were expressed as mean ± SD (n = 3).

The detailed information of Micro-BCA™ is as follows: Micro-BCA™ is a detergent-compatible bicinchoninic acid formulation for the colorimetric detection and quantitation of total protein. The method uses bicinchoninic acid (BCA) as the detection reagent for Cu^{1+}, which is formed when Cu^{2+} is reduced by protein in an alkaline environment. A purple-colored reaction product is formed by the chelation of two molecules of BCA with one cuprous ion (Cu^{1+}). This water-soluble complex exhibits a strong absorbance at 562 nm that is linear with increasing protein concentrations.

1.5.2. Dynamic histone adsorption

To investigate the adsorption kinetics of histones by microspheres, an initial concentration of 300 μg/mL was selected for the histone time-dynamic adsorption assay. 200 mg of the RCHMs was incubated with 1 mL of the histone solution at 37 ℃. The concentrations of histones were measured at 5, 10, 15, 30, 60, 90, 120, 180, 240, 300, 360, and 480 min by the Micro BCA™ protein assay reagent kit and the adsorption capacity of histones by the RCHMs was calculated according to Equation S3. The results were expressed as mean ± SD (n = 3).

The histone adsorption kinetics results of RCHMs were then fitted with pseudo-first-order (Equation S4) and pseudo-second-order (Equation S5) adsorption kinetics models:

157 $$\ln(q_e - q_t) = \ln q_{e,cal,1} - k_1 t \quad \text{(S4)}$$

158 $$\frac{t}{q_t} = \frac{1}{k_2 q_{e,cal,2}^2} + \frac{t}{q_{e,cal,2}} \quad \text{(S5)}$$

159 where q_e (μg/mg) is the experimental equilibrium histone adsorption capacity; q_t
160 (μg/mg) is the histone adsorption amount at time t; $q_{e,\ cal,1}$ (μg/mg) and $q_{e,\ cal,2}$ (μg/mg)
161 are the equilibrium histone adsorption calculated by pseudo-first-order and pseudo-
162 second-order models, respectively; and k_1 (min^{-1}) and k_2 (g min/mg) are the rate
163 constants of the pseudo-first-order and pseudo-second-order models, respectively. The
164 fitting parameters of the pseudo-second-order model can be used to calculate the initial
165 adsorption rate (h) using Equation S6.

166 $$h = k_2 q_{e,cal,2}^2 \quad \text{(S6)}$$

167 where h (mg g^{-1} min^{-1}) is the initial adsorption rate calculated by the pseudo-second-
168 order model, k_2 (g min^{-1} mg^{-1}) is the rate constant of the pseudo-second-order model,
169 and $q_{e,\ cal,2}$ (μg/mg) is the equilibrium histone adsorption amount calculated by the
170 pseudo-second-order model.

171 To investigate adsorption isotherms, histones were added to PBS to prepare histone
172 solutions with concentrations of 100, 200, 300, 600, 900, 1200, 1800, and 2400 μg/mL.
173 Then, 200 mg of the RCHMs was loaded into 1 mL of the histone solution and shaken
174 at 37 ℃. By the conclusion of the previous experiment, equilibrium was reached in the
175 adsorption process at 4 h. At this point, the supernatant absorbance was measured by
176 the Micro BCA™ protein assay reagent kit at 562 nm to calculate the equilibrium
177 concentration (C_e) using a standard curve. Then, the equilibrium histone adsorption (q_e)
178 was obtained using Equation S3.

The isotherm results were fitted using the Langmuir and Freundlich adsorption isotherm models with linear and nonlinear forms (Equations S7-10).

$$\text{linear Langmuir model: } \frac{1}{q_e} = \frac{1}{q_m K_L C_e} + \frac{1}{q_m} \quad \text{(S7)}$$

$$\text{nonlinear Langmuir model: } q_e = \frac{q_m K_L C_e}{1 + K_L C_e} \quad \text{(S8)}$$

$$\text{linear Freundlich model: } \ln q_e = \frac{1}{n} \ln C_e + \ln K_F \quad \text{(S9)}$$

$$\text{nonlinear Freundlich model: } q_e = K_F C_e^{1/n} \quad \text{(S10)}$$

where q_e (mg/g) is the histone adsorption amount (mg) per unit amount (g) of the RCHMs at equilibrium, C_e (μg/mL) is the equilibrium histone concentrations, q_m (mg/g) is the maximum adsorption capacity calculated by the Langmuir model; K_L (mL/μg) and K_F ((μg/mg) (mL/μg)$^{1/n}$) are the Langmuir and Freundlich constants, respectively; *1/n* is the constant which incorporate factors affecting the adsorbed amount at equilibrium.

1.6. Static BSA adsorption

The amount of adsorbed albumin was measured by Ultraviolet spectrophotometry (UV-1750, Shimadzu Co., Ltd, Japan) and Micro BCA™ protein assay reagent kit. To test the resistance to protein contamination of the RCHMs, BSA was added to PBS to prepare BSA solutions of 300 μg/mL and 1000 μg/mL. Then, 200 mg of the RCHMs was added into the BSA solution and shaken at 37 ℃ for 4 h. The BSA concentration was measured, and the BSA adsorption capacity was calculated according to Equations S3. The results were expressed as mean ± SD (n = 3).

2. RESULTS AND DISCUSSION

2.1. Synthesis and characterizations of the microspheres

Table S2. Analysis of elemental content in different raw materials.

Raw materials	C (wt.%)	S (wt.%)	N (wt.%)	H (wt.%)
PES	62.0	13.8	0	3.5
VP	64.8	0	12.6	8.2
AA	50.0	0	0	5.6
AMPS	40.6	15.5	6.8	6.3

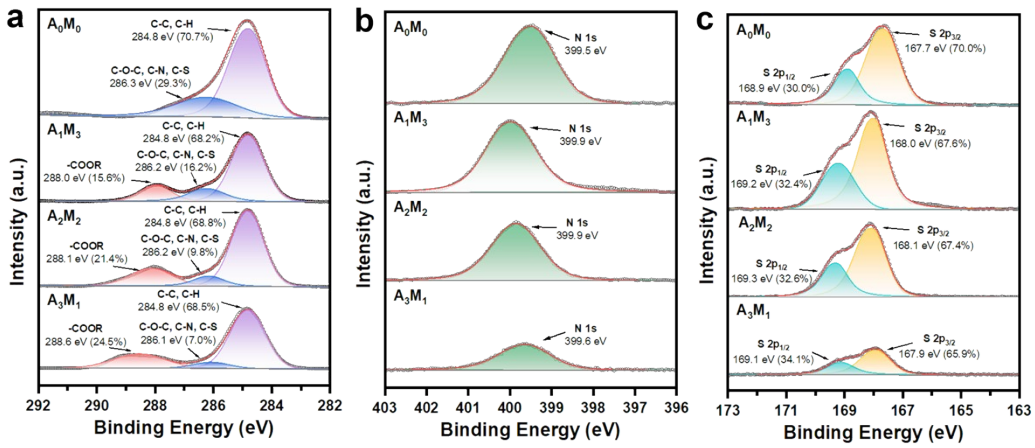

Figure S1. (a) C 1s (b) N 1s and (c) S 2p fitted high-resolution XPS spectra of the microspheres.

2.2. Histone adsorption performance

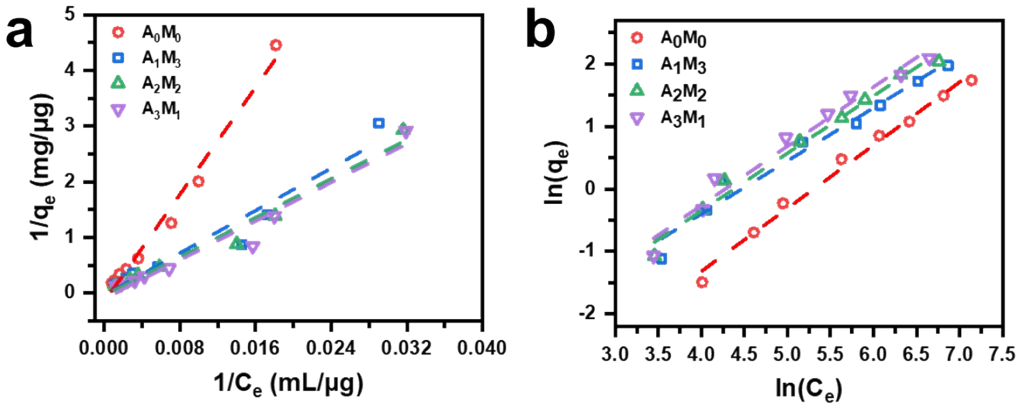

Figure S2. Adsorption isotherms of the RCHMs toward histones. (a) linear Langmuir and (b) linear Freundlich model fitting plots.

Table S3. Adsorption isotherm parameters of the microspheres toward histones.

Samples	nonlinear Langmuir			nonlinear Freundlich		
	q_m (μg/mg)	K_L (mL/μg)	R^2	n	K_F (μg/mg) $(mL/μg)^{1/n}$	R^2
A_0M_0	26.71	2.15125E-4	0.99771	1.13264	0.01055	0.99676
A_1M_3	21.78	5.09257E-4	0.99036	1.26448	0.03167	0.99395
A_2M_2	21.46	6.70346E-4	0.99330	1.26277	0.03801	0.98793
A_3M_1	20.47	8.35232E-4	0.99551	1.28153	0.04583	0.99212

2.3. Inhibition of extracellular histone-induced platelet aggregation by RCHMs

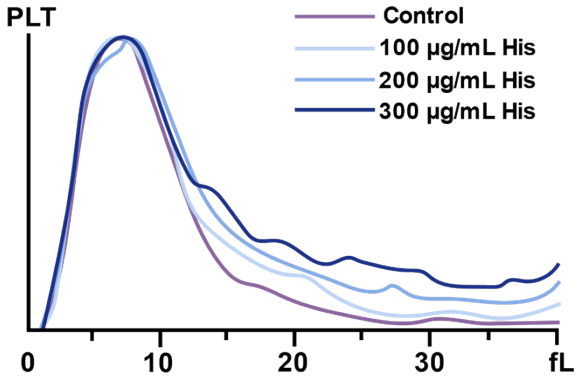

Figure S3. Platelet volume distribution histogram in histone-treated whole blood samples.