Electronic Supplementary Material (ESI) for ChemComm.
This journal is © The Royal Society of Chemistry 2025

Nitrogen-rich hyper-cross-linked polymers for rapid and selective bilirubin removal in hemoperfusion

Hongxiang Yin [a,b] , Yueyuan Xiao [a,b], Yucheng Cai [a,b], Chang Gao [a,b], Jiarui Hu[b,c] , Bien Tan *[b,c] , Irshad Hussain[d], Wei Yan* [a,b]

a. Key Laboratory of Green Preparation and Application for Functional Materials, Hubei Key Laboratory of Polymer Materials, School of Materials Science and Engineering, Hubei University, Wuhan, 430062, China.

E-mail: willieyan2003@hubu.edu.cn

b. Shenzhen Huazhong University of Science and Technology Research institute, Shenzhen, 518038, China.

c. Key Laboratory of Material Chemistry for Energy Conversion and Storage Ministry, Hubei Key Laboratory of Material Chemistry and Service Failure, School of Chemistry and Chemical Engineering, Huazhong University of Science and technology, Wuhan, 430074, China.

E-mail: bien.tan@mail.hust.edu.cn

d. Department of Chemistry and Chemical Engineering, SBA School of Science and Engineering, Lahore University of Management Sciences (LUMS). DHA, Lahore 54792, Pakistan.

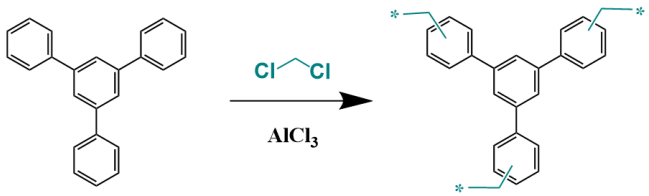
E-mail: ihussain@lums.edu.pk

1. Materials

All the reagents were purchased from commercial suppliers. Benzene, biphenyl, 1,3,5-triphenyl benzene and benzotriazole were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. Aluminum chloride ($AlCl_3$), dichloromethane (CH_2Cl_2), methanol (CH_4O), ethanol (C_2H_6O), sodium hydroxide (NaOH) and sodium chloride (NaCl) were purchased from National Medicines Corporation Ltd. of China. Bilirubin, bovine serum album and phosphate buffer solution (PBS) were purchased from Beijing Solarbio Science & Technology Co., Ltd.

2. Experimental section

2.1 Synthesis of HCP-1

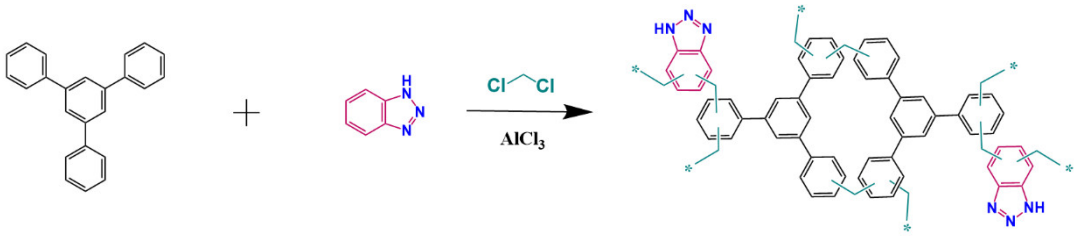


1,3,5-triphenyl benzene (0.765 g，0.0025 moL) and dichloromethane (DCM, 10 mL) were sequentially added to a 50mL single-neck round-bottom flask under nitrogen atmosphere. The reaction flask was immersed in an ice-water bath (0 °C) and the mixture was stirred magnetically until complete dissolution. Aluminum chloride ($AlCl_3$) (10.6 g, 0.080 mol) was then added. The reaction mixture was stirred at 0 °C for 12 hours. Subsequently, the temperature was gradually raised to 30 °C and maintained for 12 hours, then to 40 °C for 12 hours, followed by 60 °C for 12 hours, and finally to 80 °C for 24 hours.

Upon reaction completion, the solid product was cooled to room temperature and quenched with methanol. The solid was filtrated and sequentially washed with deionized water and methanol. The product was then purified by Soxhlet extraction with methanol for 24 hours. Finally, the solid was dried under vacuum at 80 °C for 24

hours to obtain dark brown powder sample.

2.2 Synthesis of HCP-2



1,3,5-triphenyl benzene (0.765 g，0.0025 moL) , benzotriazole (BTA, 0.864 g，0.0075 mol) and dichloromethane (DCM, 10 mL) were sequentially added to a 50mL single-neck round-bottom flask under nitrogen atmosphere. The reaction apparatus was immersed in an ice-water bath (0 °C) and the mixture was stirred magnetically until complete dissolution. Aluminum chloride ($AlCl_3$) (10.6 g, 0.080 mol) was then added. The reaction mixture was stirred at 0 °C for 12 hours. Subsequently, the temperature gradually raised to 30 °C and maintained for 12 hours, then to 40 °C for 12 hours, followed by 60 °C for 12 hours, and finally to 80 °C for 24 hours.

Upon reaction completion, the solid product was cooled to room temperature and quenched with methanol. The solid was filtrated and sequentially washed with deionized water and methanol. The product was then purified by Soxhlet extraction with methanol for 24 hours. Finally, the solid was dried under vacuum at 80 °C for 24 hours to obtain dark brown powder sample.

2.5 Procedure for bilirubin adsorption experiments

Bilirubin was initially dissolved in a small volume of 0.1 M NaOH solution, and then diluted with phosphate buffer saline (PBS, pH = 7.4) to get the desired concentration. In order to avoid the degradation of bilirubin, solution preparation and adsorption experiments were conducted under dark conditions. All the adsorption experiments

were performed in a thermostat water bath at 37 °C.

Bilirubin adsorption kinetics. 5 mg of the adsorbents were added into 10 mL bilirubin solution (100 mg/L), 9 sets in total. The mixture was shaken for 2 h at 37 °C. At appropriate time intervals, the specific set was taken and 1 mL aliquots were obtained and filtrated through a syringe filter (0.45 μm membrane filter). Then, the remaining bilirubin concentration was determined by ultraviolet spectrophotometer. The adsorption capacity (q) and removal rate (R%) of bilirubin by the adsorbent was calculated based on the following equations:

$$q_t = \frac{(c_0 - c_t)v}{m}$$

$$R(\%) = \frac{(c_0 - c_t)}{c_0} \times 100\%$$

where C_0 and C_t (mg/L) are the initial and the concentration of bilirubin at t time in the solution, respectively. V (L) is the volume of the solution, and m (g) is the mass of the adsorbent.

The kinetics data were further analyzed by the pseudo-first-order and pseudo-second-order kinetic models. Their linear equations are listed as follows:

$$ln(q_e - q_t) = lnq_e - K_1 t$$

$$\frac{t}{q_t} = \frac{1}{K_2 q_e^2} + \frac{t}{q_e}$$

where q_t and q_e (mg/g) are the adsorption capacity at time t and equilibrium time, respectively. K_1(min^{-1}) is the pseudo-first order model rate constant. K_2($g.mg^{-1}min^{-1}$) is the pseudo-second order model rate constant.

Bilirubin adsorption isotherms. 5 mg of the adsorbents was added into 10 mL bilirubin solutions with concentrations ranging from 250 to 500 mg/L. After 2h adsorption, the solution was filtered through a 0.45 μm syringe filter. The remaining bilirubin concentrations were analyzed and the adsorption capacities were calculated. The isotherm data were then analyzed by Langmuir and Freundlich isotherm models. Their linear equations are listed as follows:

$$\frac{C_e}{q_e} = \frac{C_e}{q_{max}} + \frac{1}{(K_L q_{max})}$$

$$\ln q_e = lnK_F + \frac{lnC_e}{n}$$

where q_e is the equilibrium adsorption capacity (mg/g), C_e is the equilibrium concentration (mg/L), and q_{max} and K_L are Langmuir constants related to maximum adsorption capacity and binding energy, respectively; K_F and n are empirical constants that indicate the Freundlich constant and heterogeneity factor, respectively.

Bilirubin adsorption of HCP-2 in the presence of BSA. 5 mg of HCP-TPB was added into 10 mL bilirubin solution (200 mg/L) with different bovine serum albumin concentration (11.3 mg/mL and 22.6 mg/mL). Further procedure and analysis are the same as adsorption kinetics.

Reusability tests for HCP-2. 20 mg adsorbent was added into 40 mL bilirubin solution (100 mg/L). The mixture was shaken for 2 h at 37 °C. After adsorption equilibrium, the adsorbent was filtered and was regenerated by 0.1 M NaOH solution. After washing thoroughly with deionized water and ethanol to neutral pH, the adsorbent was dried in vacuum and then reused in adsorption experiments. The experimental protocol was repeated five times.

Dynamic adsorption simulation of HCP-2. 50 mg adsorbent was added into simulated hemoperfusion column. 100 mL bilirubin solution (100 mg/L) was added into a beaker and pumped down-flow through the adsorbent column circularly by a peristaltic pump at a constant flow rate of 94.9 mL/min. At appropriate time intervals, 1 mL aliquots were obtained and filtrated through a syringe filter (0.45 μm membrane filter). Then, the remaining bilirubin concentration was analyzed and the removal rate were calculated.

2.6 Biocompatibility tests

Hemolysis assay. Hemolytic ability was determined by incubating the HCP-2 powders with red blood cells at 37 °C for 1 h. 1 mL rat blood was placed in the centrifuge tube and centrifuged at 3000 rpm for 10 min. The upper layer of serum was discarded and the blood cells in under layer were washed multiple times and then diluted with PBS solution. HCP-2 materials were washed with PBS and then diluted in

different concentrations (25, 50, 75, 100 and 125 μg/mL). The solutions were added into the suspension above subsequently. Deionized water and PBS solution were used as positive and negative control, respectively. After incubation at 37 °C for 1 h, the mixture was centrifuged at 3000 rpm for 10 min and the absorbance of the supernatant solution at 541 nm was recorded by ultraviolet spectrophotometer. The hemolysis rate was calculated using the following equation:

$$Hemolysis\ rate(\%) = \frac{A_s - A_{nc}}{A_{pc} - A_{nc}} \times 100\%$$

in which As, A_{pc} and A_{nc} are the absorbances of the sample, positive control, and negative control, respectively.

Cell viability. The cells (HEK293) were grown in Dulbecco's Modified S12 Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin-streptomycin) at 37 °C in a humidified incubator containing 5% CO_2. A standard MTT assay was used to assess the cytotoxicity of HCP-2. HCP-2 powders were sterilized with ethanol immersion and UV radiation. Cells at required density (1×10^5 cells $well^{-1}$) were seeded in 96 well plate and treated with HCP-2 solutions at varied concentrations (25, 50, 75, 100 and 125 μg/mL). Soon after treatment, plates were incubated for 24 h under above conditions. After washing with PBS thoroughly, 25 μL of MTT was added into the culture medium and the resulted solution was incubated at 37 °C for 4 h. The negative control group was incubated with 200 μL culture medium DMEM instead. Cell viability was determined by measuring the optical density at 570 nm on a microplate reader (Multiskan MK3) and calculated based on the following formula:

$$Cell\ viability\ (\%) = \frac{A_s}{A_{nc}} \times 100\%$$

where A_s and A_{nc} are the absorptions at 570 nm for the experimental and negative control wells, respectively.

3. Supporting figures

Table S1. Elemental analysis of HCP-2

Sample	C %	N %	H %
HCP-2	66.8	1.93	5.11

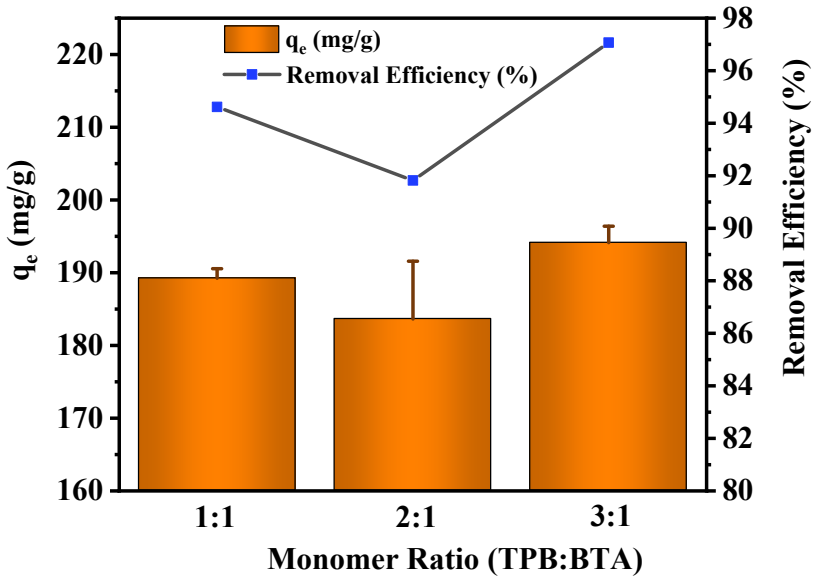


Figure S1. Adsorption performance of HCP-2 with different monomer ratios.

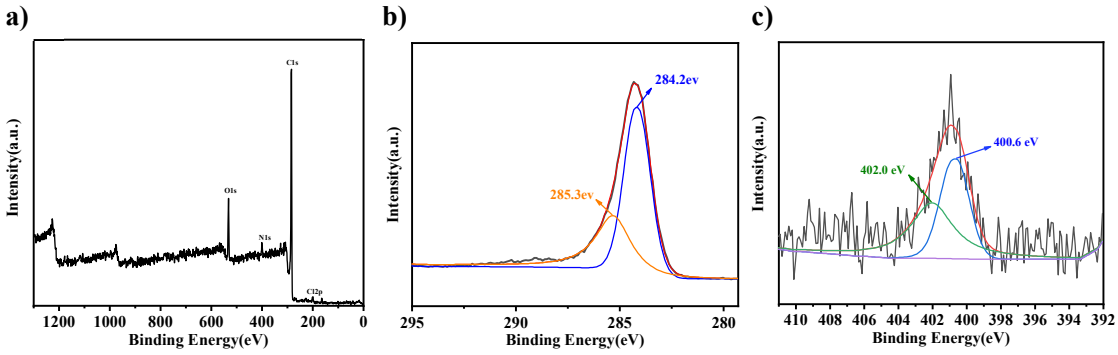


Figure S2. XPS spectrum (a) and C1s (b) and (c) N1s XPS spectrum of HCP-2

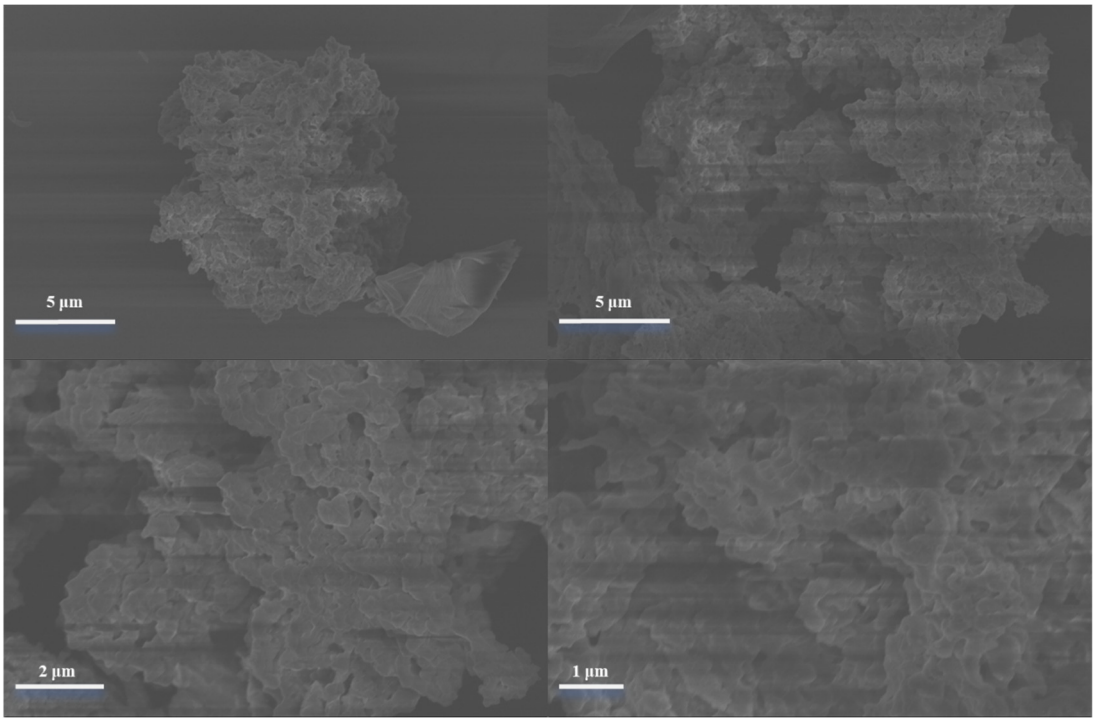


Figure S3. SEM images of HCPs.

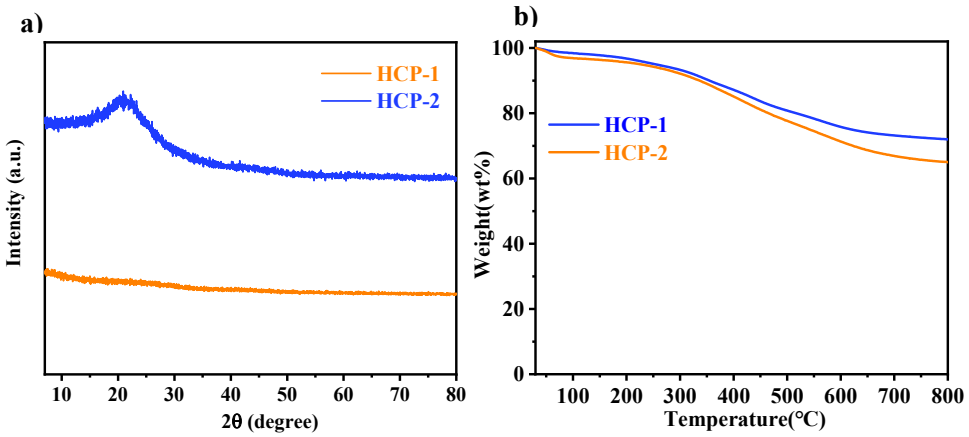


Figure S4. PXRD patterns (a) and TGA curves (b) of HCPs

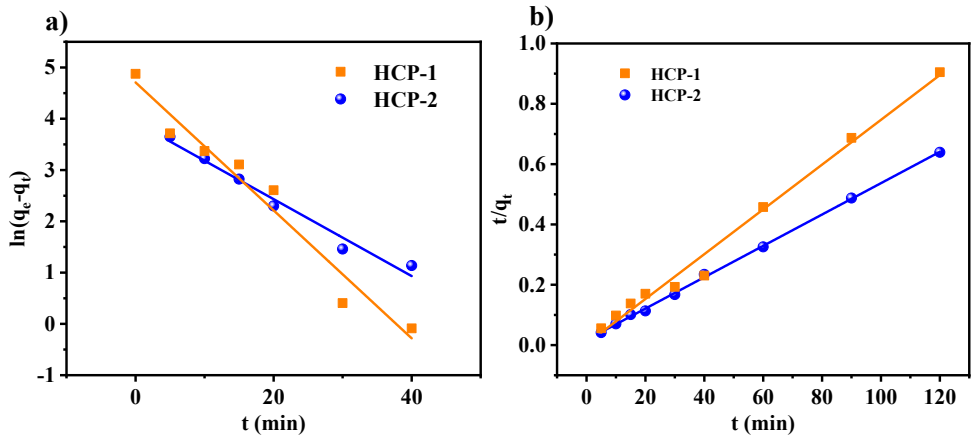


Figure S5. Fitting of pseudo first (a) and pseudo second (b) kinetic model.

Table S2. Parameters of kinetic model fitting.

Sample	Pseudo First Kinetic Model		Pseudo Second Kinetic Model		
	K_1 (min^{-1})	R^2	q_e (mg/g)	K_2 ($g.mg^{-1}min^{-1}$)	R^2
HCP-1	0.1591	0.9013	136	2.96×10^{-3}	0.9992
HCP -2	0.0267	0.9327	196	2.53×10^{-3}	0.9997

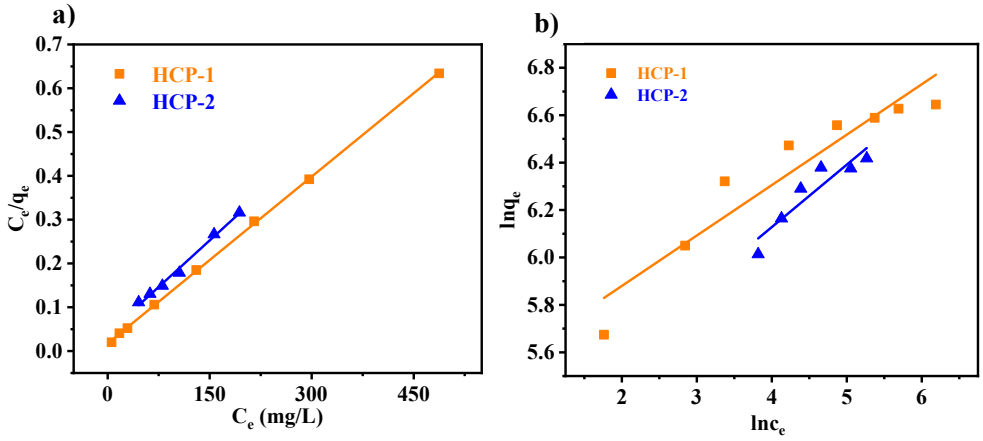


Figure S6. (a) Langmuir isotherm model and (b) Freundlich isotherm model fittings of HCPs

Table S3. Parameters of isotherm model fitting.

Sample	Langmuir Isotherm Model			Freundlich Isotherm Model		
	q_m(mg/g)	b(L/mg)	R^2	K_F	n	R^2
HCP-1	787	0.0839	0.9997	234	4.70	0.8831
HCP -2	877	0.0283	0.9965	140.2	3.04	0.9946

Table S4. Pore parameters of HCPs.

Sample	BET Surace Area (m^2/g)	Micropore Surface Area (m^2/g)	Pore Volume (cm^3/g)	Micropore Volume (cm^3/g)
HCP-1	2674	1022	2.18	0.43
HCP -2	1496	434	1.70	0.22

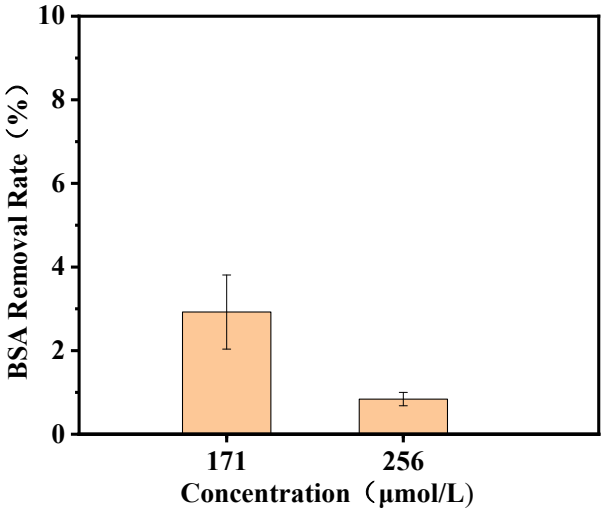


Figure S7. BSA removal efficiency of HCP-2