Supporting Information

A Microfluidic Chip Integrated with Hyaluronic Acid-Functionalized Electrospun Chitosan Nanofibers for Specific Capture and Nondestructive Release of CD44-Overexpressing Circulating Tumor Cells

Mengyuan Wang, [†] Yunchao Xiao, [†] Lizhou Lin, [‡] Xiaoyue Zhu, [†] Lianfang Du, [‡] Xiangyang Shi*[†§]

[†] State Key Laboratory for Modification of Chemical Fibers and Polymer Materials, College of Chemistry, Chemical Engineering and Biotechnology and [§] Key Laboratory of Textile Science and Technology, Ministry of Education, Donghua University, Shanghai 201620, People's Republic of China.

[‡] Department of Ultrasound, Shanghai General Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200080, People's Republic of China.

Table of Contents

---

*Corresponding author. E-mail: xshi@dhu.edu.cn

Part of Experimental Section

Materials

Chitosan (Mw = 20-30 kDa), polyethylene oxide (PEO) (Mw = 1000 kDa), β-propiolactone, hyaluronic acid (HA) (Mw = 5830 g/mol) and L-cysteine ethyl ester hydrochloride (H-Cys-OEt.HCl) were purchased from J&K Scientific Co., Ltd. (Shanghai, China). 3-Mercaptopropyltrimethoxysilane coupling agent (MPTMS, > 96%) was obtained from TCI Development Co., Ltd. (Shanghai, China). 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were from Sigma-Aldrich (St. Louis, MO). N-[3-(Dimethylamino)propyl] acrylamide (DMAPA, 98%) and 2,6-bis(1,1-dimethylethyl)-4-methylpheno (BHT) were from Aladdin (Shanghai, China). Polydimethylsil oxane (PDMS) (Sylgard 184) was purchased from Dow Corning (Midland, MI). Red blood cell lysis buffer was from Biosharp Biotech. Co., Ltd. (Shanghai, China). FITC-antiCD45 body was supplied by R&D Systems, Inc. (Minneapolis, MN). Calcein AM and propodium iodide (PI) were from KeyGen Biotech. Co., Ltd. (Nanjing, China). 4’,6-Diamidino-2-phenylindole (DAPI) was from Bestbio. Co., Ltd. (Shanghai, China). Anti-cytokeratin-7 (CK-7) antibody (Alexa Fluor 568) was supplied by Abcam Trading Co., Ltd. (Shanghai, China). Dimethylsulfoxide (DMSO) and all other chemicals and solvents were acquired from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). A549 cells (a human lung carcinoma cell line), HeLa cells (a human cervical carcinoma cell line), MCF-7 cells (a human breast adenocarcinoma cell line), and U87MG cells (a human glioblastoma carcinoma cell line) were obtained from Institute of Biochemistry and Cell Biology (the Chinese Academy of Sciences, Shanghai, China). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), streptomycin and penicillin were from Hangzhou Jinuo Biomedical Technology (Hangzhou, China). Water used in all experiments was purified using a Milli-Q Plus 185 water purification system (Millipore, Bedford, MA) with a resistivity higher than

18.2 MΩ cm. Human whole blood from healthy adult volunteers and patients suffering from non-small-cell lung carcinoma and breast cancer were kindly provided by Shanghai General Hospital (Shanghai, China) after approval by the ethical committee of Shanghai General Hospital.

Synthesis of Redox-Sensitive Targeting Ligand (HA-Cys-MPTMS) Segment.

Firstly, the carboxyl groups of HA were activated by 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide/N-hydroxysuccinimide (EDC/NHS). Briefly, HA (155.47 mg) was dissolved in 10 mL of water and then EDC (383.40 mg) was added into the HA solution. After continuously stirring for 30 min at room temperature, NHS (230.18 mg) was added into the mixture solution under magnetic stirring for 2.5 h. Afterwards, H-Cys-OEt.HCl (14.85 mg, dissolved in 5 mL water) was dropwise added into the activated HA solution under stirring for 48 h. Thereafter, the raw product was purified through dialysis against water (3 L, 9 times) using a membrane with a molecular weight cut-off (MWCO) of 1000 for 3 days. The HA-Cys compound was obtained after lyophilization, and then stored at 4 ^{o}C for subsequent use.

Afterwards, HA-Cys-MPTMS containing cleavable disulfide bond was synthesized. To be specific, HA-Cys compound (32 mg) was dissolved in H_2O_2 (5 mL, 30%) and then 10 μL of 3-mercaptopropyltrimethoxysilane (MPTMS) coupling agent was dropwise added into the reaction system under an ice bath, followed by magnetic stirring for 4 h. Then, the raw product was purified through dialysis against water (3L, 9 times) with a membrane having an MWCO of 1000 for 3 days. After lyophilization, the final product of HA-Cys-MPTMS was obtained.

Synthesis of CBAA

Zwitterion of carboxyl betaine acrylamide (CBAA) was first synthesized according to the literature.[1] In brief, N-[3-(Dimethylamino)propyl] acrylamide (DMAPA) (1.00 g) and β-propiolactone (0.64 g) were co-dissolved in anhydrous acetone (10 mL) and reacted at 0 ^{o}C for 3 h

under nitrogen protection. The CBAA was obtained by washing the white precipitate with anhydrous acetone and anhydrous ether, followed by drying in vacuum, and stored at 4 °C. ^{1}H NMR (in D_2O): 6.11 (t, 1H, CHH=CH), 6.05 (t, 1H, CHH=CH), 5.62 (t, 1H, CHH=CH), 3.40 (t, 2H, N-CH_2-CH_2-COO), 3.20 (m, 4H, NH-CH_2-CH_2-CH_2), 2.91 (s, 6H, N-$(CH_3)_2$), 2.49 (t, 2H, C_2-COO), 1.89 (t, 2H, NH-CH_2-CH_2-CH_2).

Fabrication of Water-Stable Electrospun CNFs

Chitosan/polyethylene oxide (PEO) (mass ratio = 9:1) solution (3.0 wt%) was prepared by dissolving chitosan and PEO in 85% aqueous acetic acid under magnetic stirring overnight at room temperature until the solution reached a homogeneous and transparent state. Subsequently, the spinning dope was brought into a 10-mL syringe capped with a needle (with an inner diameter of 0.6 mm). The parameters of electrospinning were set as follows: the feeding rate was fixed to 0.3 mL h^{-1} using a syringe pump (model 78-9100C, Cole Palmer Instrument Co., New York, NY); the collecting distance was set to be 12 cm; the voltage was set at 30 kV; and the electrospinning process was conducted in an ambient environment (30%-40% humidity and 20-25 °C). The nanofibers were collected either on circular cover slips (14 mm in diameter) or glass slides (25 mm × 75 mm). The formed chitosan nanofiber (CNF) mats were then crosslinked by glutaraldehyde (GA) vapor to crosslink the PEO hydroxyl and chitosan amine/hydroxyl groups in a vacuum desiccator for 6 h to render them with water stability according to the literature.[2] The crosslinked nanofibrous mats were then dried in vacuum at an ambient temperature for 24 h before use.

Characterization

^{1}H NMR measurement was carried out on a Bruker DRX 500 NMR spectrometer operating at 400 MHz. D_2O was used as a solvent to dissolve all materials before measurement. The morphology of the chitosan nanofibrous mats were observed using scanning electron microscopy (SEM, JEOL

JSM-5600LV, Tokyo, Japan) with an accelerating voltage of 5 kV. The samples were sputter coated with a gold film with a thickness of 10 nm before observation. The nanofiber diameter distribution was analyzed using ImageJ software (https://imagej.nih.gov/ij/download.html), and at least 300 nanofibers from different SEM images were measured for each sample. Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy was performed on a Nicolet Nexus 670 FTIR spectrophotometer in the wavenumber range of 500-4000 cm^{-1}. The water contact angle was measured using an optical contact angle goniometer (Dataphysics OCA40Micro, Damstadt, Germany) to determine the hydrophilicity of the chitosan nanofibrous mats before and after modification. Water drops (3 μL each) were placed onto the fibrous mat at three different locations (one drop for each location) before measurements. Thermal gravimetric analysis (TGA) was performed using a TG209 F1 thermogravimetric analyzer (NETZSCH Instruments Co., Ltd., Selb/Bavaria, Germany) under nitrogen atmosphere with a temperature range of 30-900 ^{o}C.

Hemocompatibility Assay

The hemocompatibility of the CNFs, CBAA-CNFs and CBAA-CNFs-HA was evaluated *via* hemolysis and dynamic clotting assays according to protocols described in the literature.[3] Human whole blood (stabilized with heparin) from healthy adult volunteers were used for hemocompatibility tests. For hemolysis assay, the whole blood were centrifuged and washed with PBS to completely remove serum and obtain red blood cells. CNFs, CBAA-CNFs, and CBAA-CNFs-HA with a mass concentration of 4 mg mL^{-1} were tested, respectively. For dynamic clotting assay, the whole blood was used and the concentration of the nanofibrous mat was also set at 4 mg mL^{-1} for different fiber types.

Pretreatment of the Whole Blood

Human whole blood (collected in anticoagulative tubes) from healthy adult volunteers or

patients was obtained from Shanghai General Hospital (Shanghai, China) and used after approval by the Shanghai General Hospital Ethical Committee. All related experiments were performed in compliance with the relevant laws and guidelines of Shanghai General Hospital and the policy of the National Ministry of Health. Human whole blood was first lysed using red blood cell (RBC) lysis buffer to get rid of RBCs. Subsequently, the mixture was centrifuged (1000 rpm, 5 min) and rinsed three times with phosphate buffered saline (PBS) to move residual lysis buffer, and thus RBC-lysed blood was obtained for further study.

Design and Fabrication of the Functionalized Fiber-Integrated Microfluidic Chip

The microfluidic chip was designed by Auto CAD and then printed in a photographic film using a high resolution printer. In detail, the chip had a single inlet and outlet. An array of 277 elliptical columns (major axis was 1.0 mm and minor axis was 0.5 mm) were designed in the microfluidic channel for supporting the polydimethylsil oxane (PDMS) chip and distributing the flow. The height, length and width of the microfluidic chip were 50 μm, 50 mm, and 7 mm, respectively.

A traditional soft lithographic process was utilized to fabricate the microfluidic channel on a silicon wafer. After reverse mould, a transparent PDMS microfluidic channel was obtained. Lastly, the glass slide covered with CBAA-CNF-HA mats was bonded with the PDMS channel *via* plasma treatment.

Statistical Analysis

One-way ANOVA statistical method was adopted to analyze the experimental results. A value of 0.05 was selected as the significance level, and the data were marked with (*) for $p < 0.05$, (**) for $p < 0.01$, and (***) for $p < 0.001$, respectively.

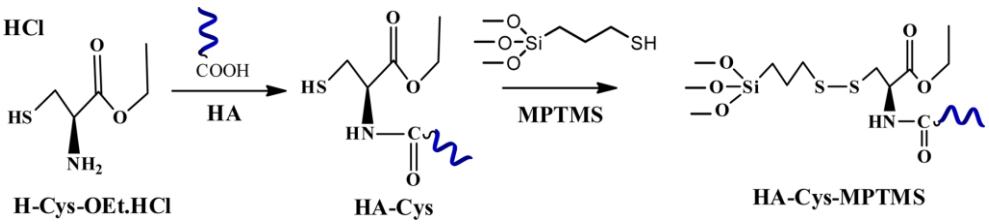

Figure S1. Schematic illustration of the synthesis of HA-Cys-MPTMS segments.

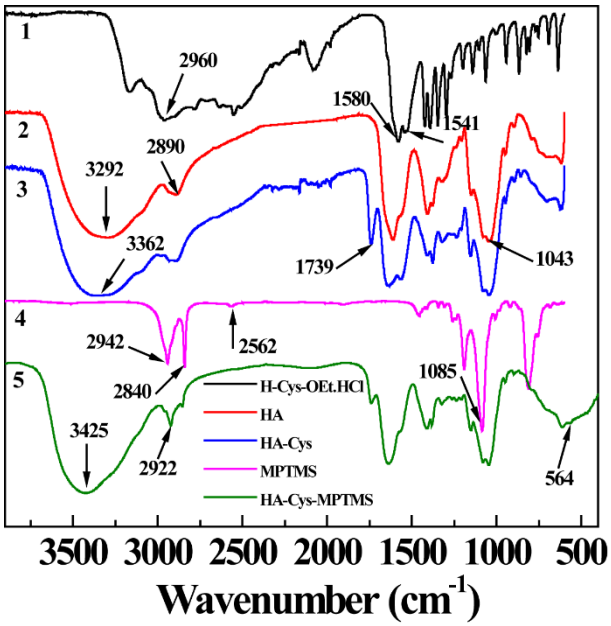

Figure S2. FTIR spectra of H-Cys-OEt.HCl (1), HA (2), HA-Cys (3), MPTMS (4), and redox-sensitive HA-Cys-MPTMS segments (5).

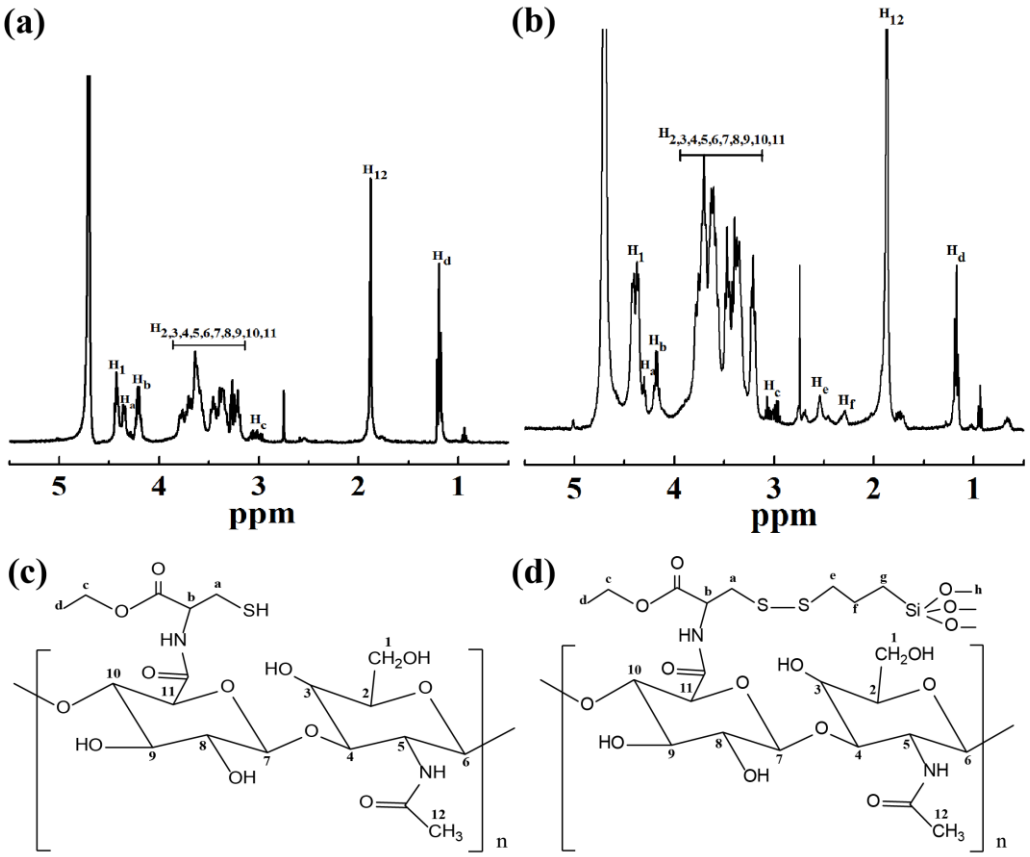

Figure S3. ^{1}H NMR spectra of HA-Cys (a) and HA-Cys-MPTMS (b) dissovled in D_2O, and the chemical structure of HA-Cys (c) and HA-Cys-MPTMS (d).

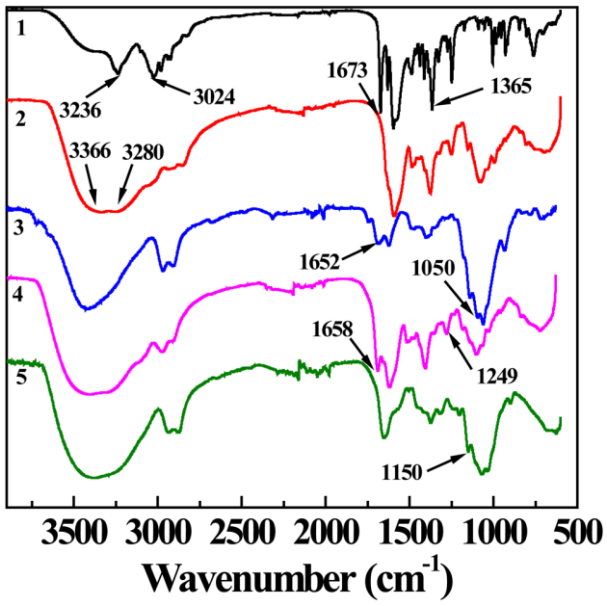

Figure S4. FTIR spectra of CBAA (1), pristine CNFs (2), GA-crosslinked CNFs (3), CBAA-CNFs (4), and CBAA-CNFs-HA (5).

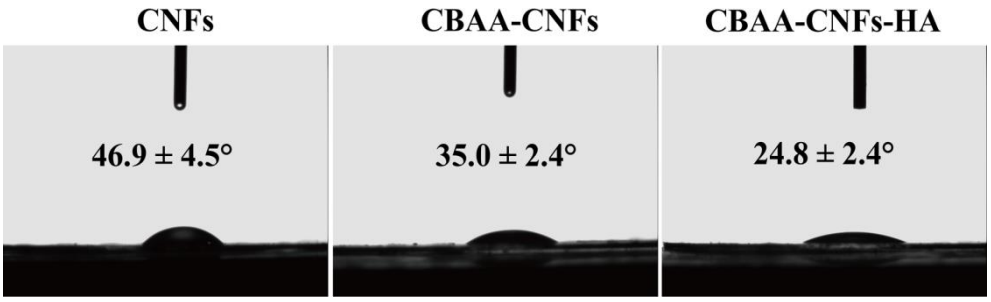

Figure S5. Water contact angles of CNFs, CBAA-CNFs, and CBAA-CNFs-HA at 5 s.

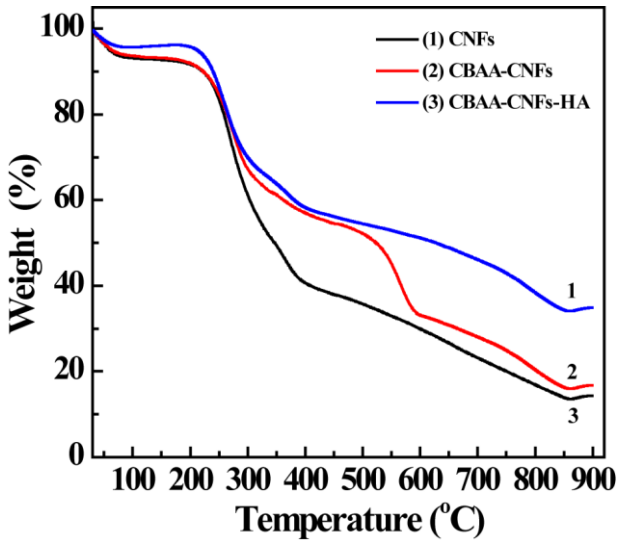

Figure S6. TGA curves of CNFs (1), CBAA-CNFs (2) and CBAA-CNFs-HA (3).

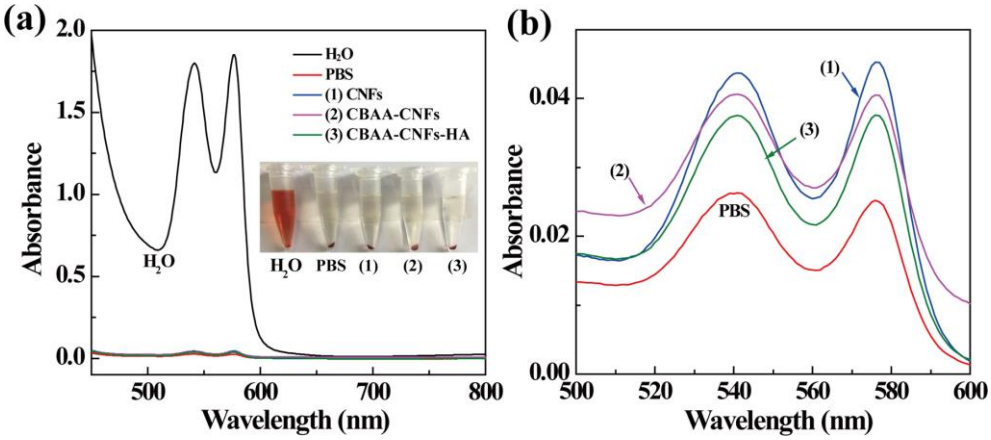

Figure S7. (a) UV-vis spectra of human red blood cell suspensions treated with H_2O, PBS, CNFs (1), CBAA-CNFs (2), and CBAA-CNFs-HA (3) for 2 h, respectively. The fiber concentration was set at 4 mg/mL for all samples. The inset shows a photograph of red blood cells exposed to water, PBS, and different nanofibers (at a concentration of 4 mg/mL), respectively followed by centrifugation. (b) Enlarged UV-vis spectra shown in (a) in a wavelength range of 500-600 nm.

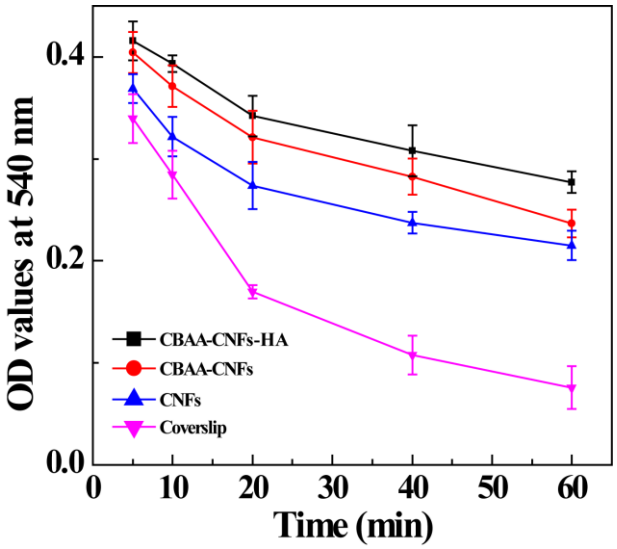

Figure S8. Anticoagulant assay of coverslip, CNFs, CBAA-CNFs, and CBAA-CNFs-HA at different time intervals.

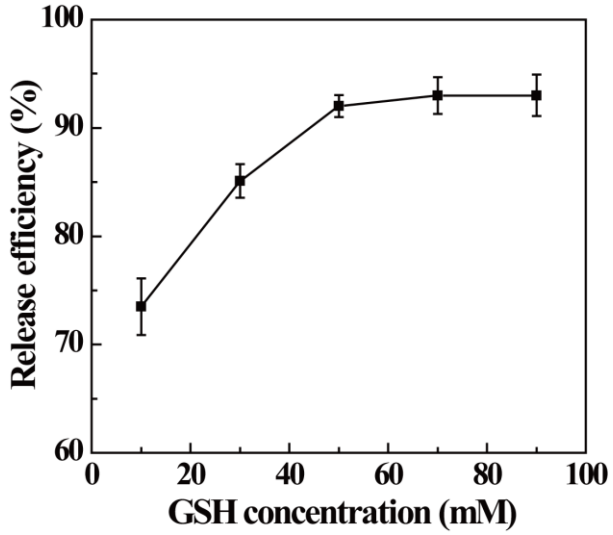

Figure S9. The release efficiency of A549 cells after incubated with different concentration of GSH for 1 h.

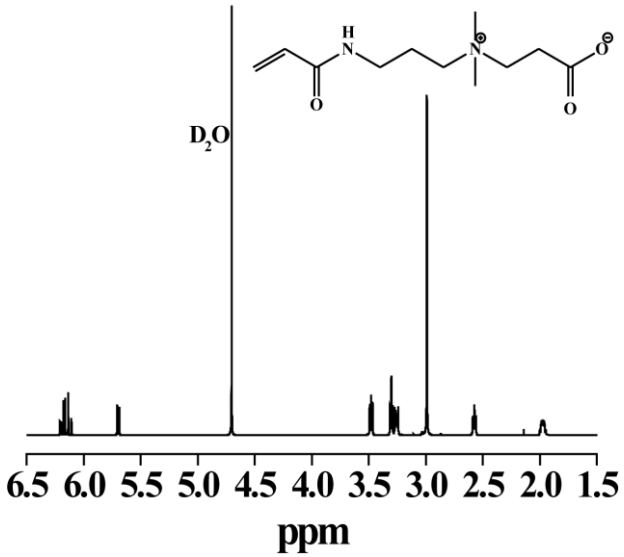

Figure S10. ^{1}H NMR spectrum of CBAA monomer dissovled in D_2O.

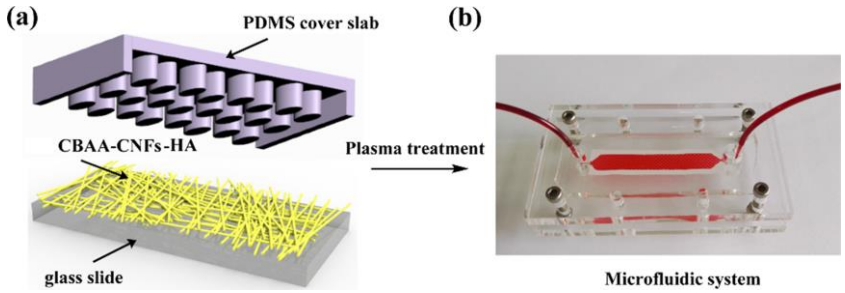

Figure S11. Design of a microfluidic chip embedded with CBAA-CNF-HA mats for capturing CD44 receptor-overexpressing CTCs: (a) a three-dimensional illustration of a microfluidic chamber and glass slide covered with CBAA-CNF-HA mats; (b) photograph of the microfluidic system for capturing CD44 receptor-overexpressing CTCs.

References

(1) Zhang, P. C., Chen, L., Xu, T. L., Liu, H. L., Liu, X. L., Meng, J. X., Yang, G., Jiang, L., and Wang, S. T. (2013) Programmable Fractal Nanostructured Interfaces for Specific Recognition and Electrochemical Release of Cancer Cells. *Adv. Mater. 25*, 3566–3570.

(2) Sun, N., Liu, M., Wang, J., Wang, Z., Li, X., Jiang, B., and Pei, R. (2016) Chitosan Nanofibers for Specific Capture and Nondestructive Release of CTCs Assisted by pCBMA Brushes. *Small 12*, 5090–5097.

(3) Zhao, Y. L., Wang, S. G., Guo, Q. S., Shen, M. W., and Shi, X. Y. (2013) Hemocompatibility of Electrospun Halloysite Nanotube and Carbon Nanotube-Doped Composite Poly(Lactic-Co-Glycolic Acid) Nanofibers. *J. Appl. Polym. Sci. 127*, 4825–4832.