Supporting Information

for

Dual-affinity peptide mediated inter-protein recognition

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Methods

Peptides

Peptide E5 (GGRSFFLLRRIQGCRFRNTVDD) was selected from a collection of chemically synthesized peptides in previous studies.[1] E5 peptide with and without fluorescein isothiocyanate (FITC) labeling used in this study (purity 98%) were both purchased from GL Biochem Ltd (Shanghai) and used without further purification.

Peptide C16 (GGTRALAFFDCGGNNNCCNNN) with and without fluorescein isothiocyanate (FITC) labeling used in this study (purity 98%) were both purchased from GL Biochem Ltd (Shanghai) and used without further purification.

Cell culture

Murine stromal cell MS-5 was kindly provided by Professor Haiyan Xu, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, China. Human breast cancer cell MDA-MB-231 was gifted by Professor Chunying Chen, National Center for Nanoscience and Technology, Beijing, China. Human promyelocytic leukemia cell HL-60 was purchased from Chinese Academy of Medical Science & Peking Union Medical College, Beijing, China. Cell MS-5 and HL-60 were cultured in a dish in RPMI-1640 (Hyclone Thermo Scientific) and cell MDA-MB-231 in DMEM culture medium (Hyclone Thermo Scientific). The culture media were

supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone Thermo Scientific) and 1% streptomycin-penicillin. The dish was placed in 5% CO_2 at 37°C in a cell culture incubator.

Preparation of E5-HSA complex

Peptide E5 and HSA (Sigma-Aldrich, USA) were dissolved in sterile water to the concentration of 1 mg/mL and 125 mg/mL, respectively. A certain amount of HSA aqueous solution was mixed into E5 aqueous solution at a molar ratio of 1:1. After fully mixed, the aqueous solution was then added into proper amount of 10×PBS (phosphate buffer solution; 80 g NaCl, 2 g KCl, 14.2 g Na_2HPO_4 and 2.7 g KH_2PO_4 dissolved in 1000 mL sterile water, pH 7.2-7.4) to prepare a solution of E5-HSA nanocomplex in 1×PBS.

Characterization of E5-HSA complex by mass spectrometry (MS)

The complex was further characterized using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) on a Microflex LRF (Bruker Daltonics, USA) to determine the molecular weight. The solution was prepared by dissolving the E5-HSA complex (the concentration of E5 is 1 mg/mL) in sterile water. 2 μL of the solution was mixed with 2 μL of matrix solution. The matrix solution used for characterizing E5-HSA complex consisted of 10 mg/mL 2,5-dihydroxybenzoic acid (DHB) in 1:1 acetonitrile/ water. The mixture was spotted on the target plate (2 μL per spot), and the solvent was allowed to

evaporate under vacuum conditions. Analysis was performed in the linear mode.

Cy5-NHS-ester labeling of HSA

The Cy5 mono N-hydroxysuccinimide ester (Cy5-NHS-ester, Lumiprobe, USA) was dissolved in amine-free dimethyformamide (DMF) to 1.5 mg/mL and HSA was dissolved in 0.1 M phosphate buffer (pH 8.3-8.5) to 2.3 mg/mL. Then 200 μL Cy5-NHS-ester solution was added into the solution of HSA. The mixed solution was kept on ice overnight. Then the solution was dialyzed in regenerated cellulose dialysis bags [with 8000-14000 molecular weight cut-off (MWCO)] against ultrapure water, followed by purifying the conjugate on a PD-10 desalting column (GE Healthcare, USA) and then a centricon device (10k MWCO). The sample collection was freeze-dried and dissolved in sterile water prior to use. Cy5 labeling of HSA was characterized by MS according to the above method.

Measurement of the binding affinity of the peptide E5 to HSA by circular dichroism (CD) spectra

CD experiments were performed with a quartz cell of 0.1 cm path length on a spectropolarimeter (J-1500, JASCO, Japan). The samples were dissolved in ultrapure water at room temperature. CD spectra of HSA, the peptide E5, and mixture solutions of E5 and HSA were recorded at 3 μM, and every CD spectrum data used in this article is the average of three scans and calibrated by the blank.

In the titration experiment, HSA was dissolved in 600 μL ultrapure water with the initial concentration of 1.5 μM, and the concentration of the peptide E5 was 120 μM. Titration was carried out with the peptide solution added to the HSA solution. In the control group, 600 μL HSA sample of 1.5 μM was titrated with the same volumes of ultrapure water as the peptide solution. The secondary structures of the mixed solutions for both control and experimental group were recorded during the titration, and the data at 222 nm were collected. The differential ellipticities $\Delta\theta_{222}$ were obtained by subtracting the control data, and the horizontal axis represents the concentrations of the peptide E5 in mixed solution. The K_D was obtained by fitting the following equation:

$$\Delta\theta_{222} = \frac{A C_H C_P}{K_D + C_P} \quad \text{(Equation S1)}$$

where C_H corresponds to the concentration of HSA, and C_P corresponds to the concentration of the peptide E5. $\Delta\theta_{222}$ represents the difference of CD intensity of the mixed solution and control at 222 nm, and A in the equation corresponds to a constant.

The binding affinity of the peptide C16 to HSA was measured as above.

Measurement of the CXCR4 expression levels of cells

The MS-5, MDA-MB-231 and HL-60 cells were washed 3 times with washing buffer (PBS containing 2% FBS) and then resuspended with washing buffer to 5×10^6 cells/mL. 50 μL of the cell suspension was added

into the tube and centrifuged. Then the supernatant was removed carefully. 60 μL of FITC-labeled CXCR4 antibody (MBL, Japan) in the washing buffer was added at 10 μg /mL. The cells were incubated for 30 minutes at room temperature. 1 mL of washing buffer was added followed by centrifugation. Then the supernatant was removed and cells were resuspended with 500 μL washing buffer. 1×10^4 cells were analyzed. FITC-labeled IgG was chosen as the control.

Measurement of the binding affinity of the E5, HSA and the complex of E5-HSA to cells by flow cytometey (FCM)

The MS-5, MDA-MB-231 and HL-60 cells were washed once with washing buffer (PBS containing 2% FBS) and then resuspended with complete medium to 5×10^6 cells/mL. 50 μL of the cell suspension was added into the Eppendorf tube and centrifuged at 500 g for 1 min. Then the supernatant was removed carefully. 60 μL of FITC-labeled E5, Cy5-labeled HSA, complex of FITC-labeled E5 and HSA, and complex of E5 and Cy5-labeled HSA at different concentrations in complete medium were added into the tube. The cells were incubated at room temperature for 1 h and then washed twice with 1 mL washing buffer. Then the supernatant was removed and cells were resuspended with 500 μL washing buffer. 1×10^4 cells were analyzed by C6 Accuri flow cytometer (BD Biosciences, USA).

The K_D was calculated according to the fitting line plotted as a function

of the concentration of the fluorescent phase by the Langmuir equation:

$$F = \frac{B_{max} C_a}{K_D + C_a} \qquad \text{(Equation S2)}$$

where F is the MFI of the cells, B_{max} is the maximal MFI of the cells, C_a is the concentration of the fluorescent phase ($mol \cdot L^{-1}$).

The binding affinity of the peptide C16 and the complex of C16-HSA to cells was measured as above.

Confocal microscopic images of MDA-MB-231 cells bound with signaling molecules

The MDA-MB-231 cells were seeded at 10×10^4 cells/well and incubated for 24 h. Then the cells were washed 3 times and 1 mL of complete medium containing 1 μM Cy5-labeled HSA or 1 μM complex of E5 and Cy5-labeled HSA was added. Cells were incubated for 1 h. Next the cells were washed and mounted with complete medium containing 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, USA). Images were photographed using a laser confocal microscope (LSM760, Zeiss, Germany).

In the colocalization assay, 1 mL of complete medium containing 0.01 μM complex of FITC-labeled E5 and Cy5-labeled HSA was added to form dispersed fluorescent signals.

Transwell assay

The transwell assay was applied using millicell hanging cell culture

inserts (8 μm; Millipore, Switzerland). MDA-MB-231 (10×10^4 cells/chamber) and HL-60 (20×10^4 cells/chamber) cells were plated into the upper chambers of the inserts in opti-MEM medium (Gibco, Thermo Fisher Scientific, USA) containing different concentrations of E5 and E5-HSA. Then the inserts were placed into a well of a 24-well plate containing 800 μL 10 % FBS medium for MDA-MB-231 and HL-60 in the presence or absence of CXCL12. The concentrations of CXCL12 were 100 ng/mL for MDA-MB-231 cells and 200 ng/mL for HL-60 cell respectively. After 24 h incubation, for MDA-MB-231 cells, the top surface of the insert was scraped using a cotton swab and the cells on the lower surface of the membrane were fixed and stained with crystal violet. Cells which had migrated to the bottom of the membrane were visualized and counted using a microscope (DMI3000B, Leica, Germany). For each replicate, cells in five randomly selected fields were counted and averaged. For HL-60 cells, cells migrating to the lower chambers were counted.

Molecular docking

The NMR structure of CXCR4 and HSA were retrieved from the RSCB Protein Data Bank (the PDB ID of CXCR4 is 3OE0 and the PDB ID of HSA is 4L9K). Structure of E5 was obtained by PyMol, and formation converted and 3D structure generated by Open Babel. Rotatable bonds within the peptide were defined through AutoDockTools. AutoDock Vina was used for automated docking simulation. Standard parameters which

were created with Raccoon, a script program in the AutoDock software package, were used. Calculations were performed with a 26-node PC cluster in which each node has a 16-core central processing unit (2.8 GHz, Pentium-D, Intel, USA). Lowest binding free energy was recorded as the binding affinity between HSA, E5 and CXCR4.

References

1 X. Li, H. Guo, Y. Yang, J. Meng, J. Liu, C. Wang and H. Xu, *Sci. Rep.*, 2014, **4**.

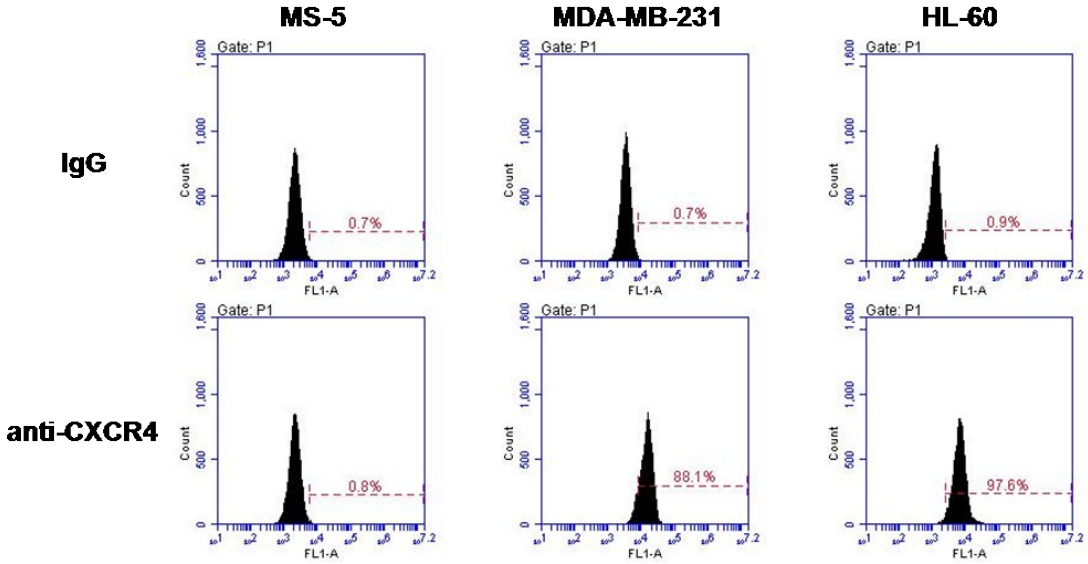

Fig. S1 Expression levels of CXCR4 in MS-5, MDA-MB-231 and HL-60 cells. Expression level of IgG was used as control. The typical results representative of three repeated experiments were shown. Results were measured by FCM analysis using FITC-labeled anti-CXCR4.

Three cell lines MS-5, MDA-MB-231 and HL-60 were chosen as cell models because the cells express different levels of CXCR4 in the surface. The CXCR4 levels for MS-5, MDA-MB-231 and HL-60 were detected 0.8%, 88.1% and 97.6% respectively. Results were shown in Fig. S1.

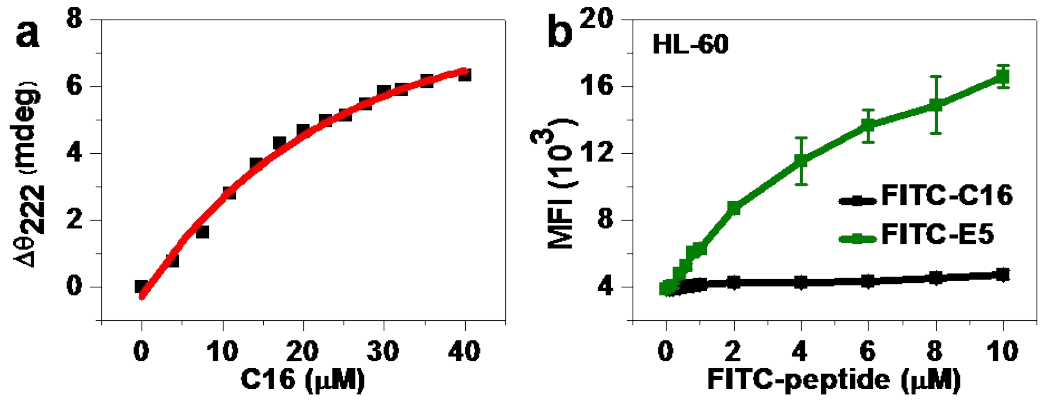

Fig. S2 Affinity of C16 to HSA and CXCR4-positive cell HL-60. (a) $\Delta\theta_{222}$ was plotted as a function of concentration of the peptide C16 for the titration of C16 interacted with HSA. The heavy lines represented the fitting lines using Equation S1 (ESI†). (a) The binding amounts of C16 to HL-60 were measured by FCM analysis using FITC as the label to C16. The result of E5 was showed as a control. Error bars represent the standard deviation (n=3).

As shown in Fig. S2, C16 manifested comparable binding affinity (2.2 μM) to that of E5 binding to HSA by fitting in MATLAB software using Equation S1. However, C16 bound little to CXCR4-positive cell HL-60 and the binding amount increased much slower within 10 μM compared with E5.

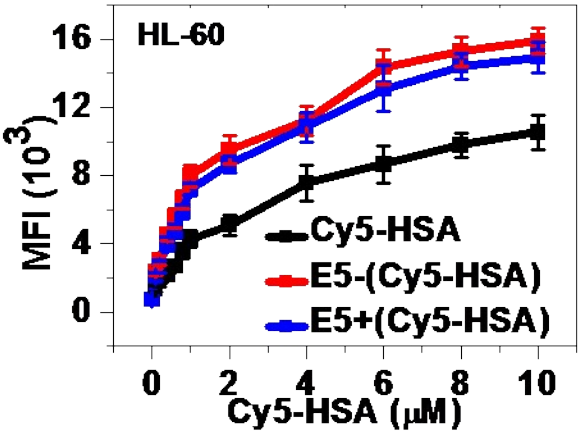

Fig. S3 Affinity of HSA to HL-60. The binding amounts of HSA to HL-60 were plotted as a function of the concentration of HSA. The black, red and blue line represented cells were exposed to free HSA, HSA bound with E5, and first E5 and then to HSA, respectively. Results were measured by FCM analysis using Cy5 as the label to HSA. Error bars represent the standard deviation (n=3).

In the experiment that cells were exposed to E5 first and then to HSA, we conducted it according to the following assay: the cells were incubated with E5 for 1 h and washed twice, and the incubated with Cy5-labeled HSA for another 1 h. As shown in Fig. S3, when cells were exposed to E5 first and then to HSA, enhanced affinity was also observed between HSA and HL-60. This result further confirmed that E5 mediated recognition between HSA and CXCR4.

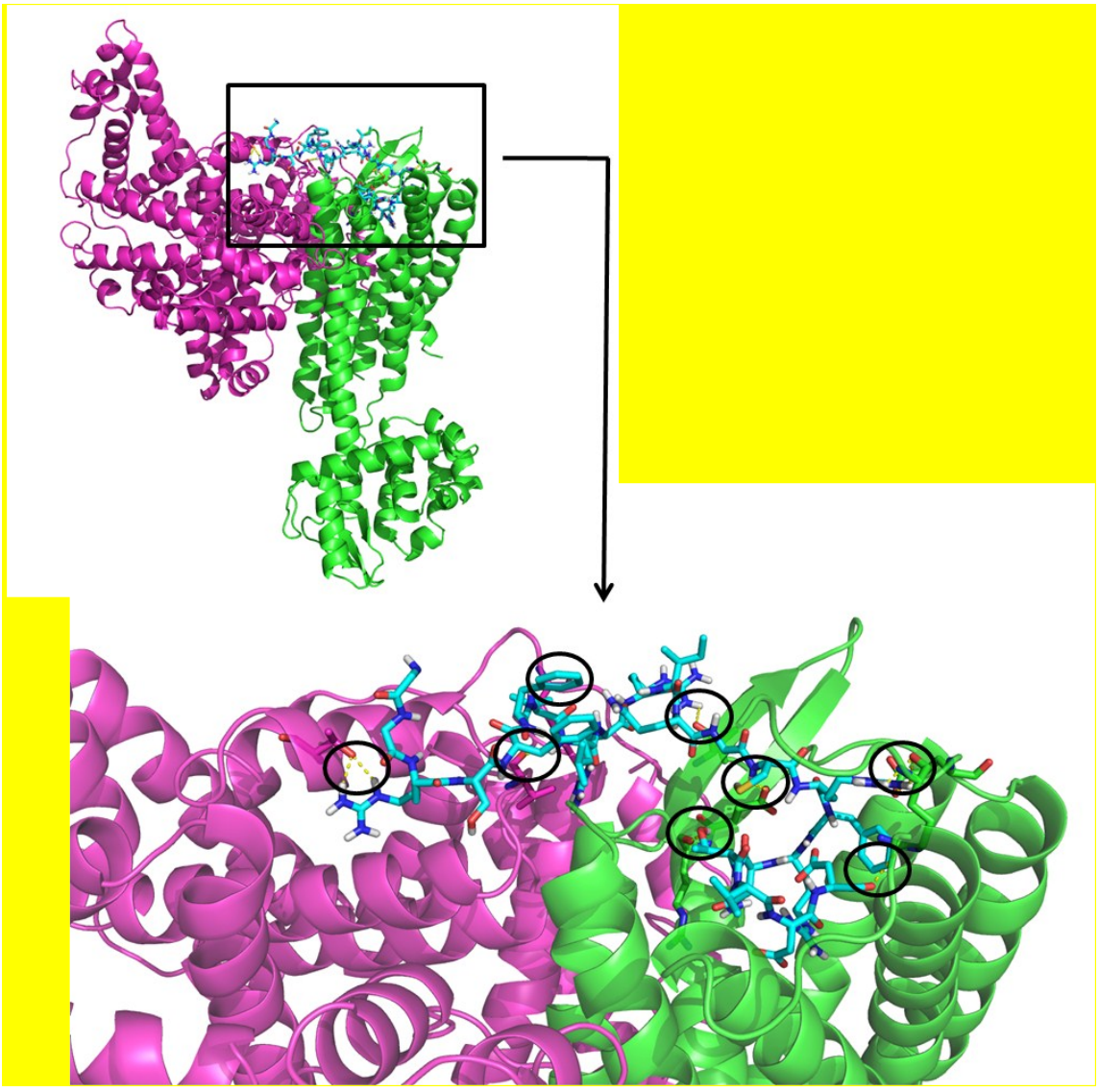

Fig. S4 Molecular modeling simulations between HSA (pink), W4 (aqua sticks) and CXCR4 (green). Zoomed-in part showed possible hydrogen bonds between Val469, Thr478 and Tyr497 in HSA and the

N-terminal domain of E5, and between Glu32, Arg38, Val177, Asp187 and Arg188 in CXCR4 and the C-terminal domain of E5 in black circles. Molecular docking studies were initiated with AutoDock Vina.

To further reveal the interaction mode between HSA, E5 and CXCR4, the automated docking simulation was performed. The crystal structure of HSA (PDB ID: 4L9K) and CXCR4 (PDB ID: 3OE0) were utilized as the target structure, whereas the peptide E5 was used as a docking probe. According to the illustration (Fig. S4), the N-terminal domain of E5 formed intermolecular hydrogen bonds with HSA at Val469, Thr478 and Tyr497, and the C-terminal domain of E5 bound with CXCR4 at Glu32, Arg38, Val177, Asp187 and Arg188.

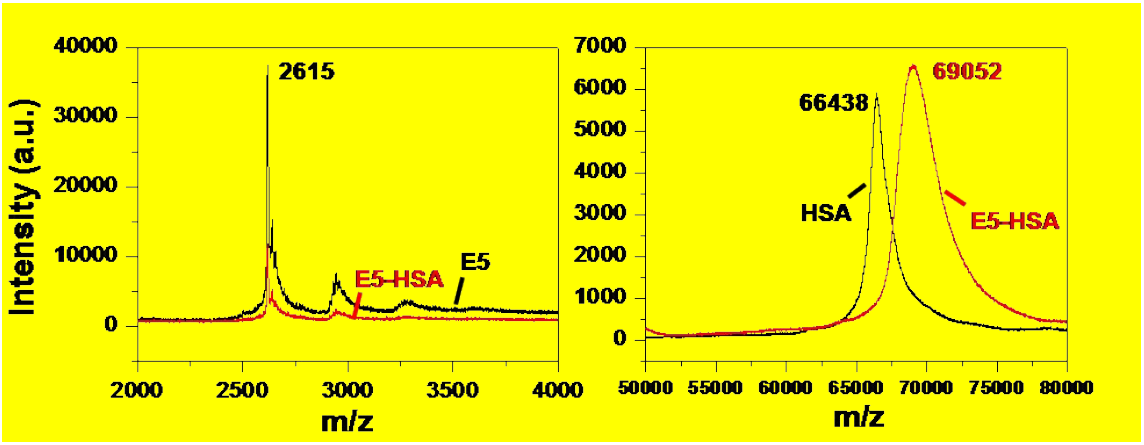

Fig. S5 MALDF-TOF MS spectra for E5 (left), HSA (right) and E5-HSA complex (the red line). The results revealed a difference in mass of HSA after binding with E5, which were consistent with the successful formation of the E5-HSA complex.

MALDI-TOF MS analysis (Fig. S5) demonstrated an increase in the molecular weight of HSA after binding with E5 [MW (HSA)=66438, MW (E5-HSA complex)=69052], and a decrease in the peak intensity of

E5, suggesting the formation of a stable E5-HSA complex.

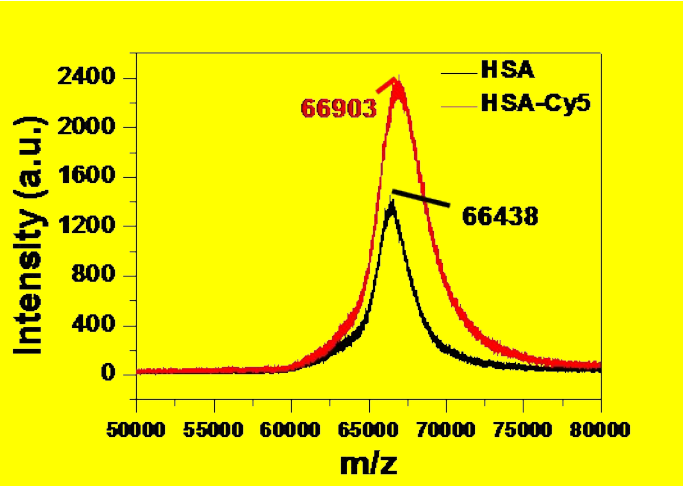

Fig. S6 MALDF-TOF MS spectra for HSA and Cy5 labeled HSA (the red line). The results revealed a difference in mass of HSA after covalent linkage with Cy5, which were consistent with the successful formation of Cy5 labeled HSA.

MALDI-TOF MS analysis (Fig. S6) demonstrated an increase in the molecular weight of HSA after covalent linkage with Cy5 [MW (HSA)=66438, MW (Cy5 labaled HSA)=66903], suggesting the formation of Cy5 labeled HSA.