Electronic Supplementary Information

for

A flavonoid-based fluorescent probe enables the accurate detection of human serum albumin by minimizing the interference from blood lipids†

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1. Materials

All biological components including human serum albumin, glutathione (GSH), cysteine (Cys), homocysteine (Hcy), dithiothreitol (DTT), ribonucleic acid (RNA), lysozyme (Lyso), globulin (Cohn fraction IV-4 human), ribonuclease (Ribo), trypsin, and lipase were purchased from Sigma-Aldrich without further purification. Glyceryl trioleate (TAG), palmitic acid (NEFA), cholesterol (CE), L-α-phosphatidylcholine (PL, from egg yolk, Type XVI-E, 99% pure), ibuprofen and warfarin were purchased from Sigma-Aldrich and used as received. All chemicals and solvents were purchased from Energy Chemical China without further purification. The serum samples were provided from Longhua Central Hospital in Shenzhen, and the ethical approval was also given by the medical ethics committee of Longhua Central Hospital.

2. Instruments

^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker AVANCE III 600-MHz spectrometer. The high-resolution mass spectra (HRMS) were measured by Thermo-Fisher Q Exactive spectrometer (ESI-TOF). UV-Vis spectra were obtained by a Thermofisher Evolution 220. The fluorescence spectra were measured using a Thermo Lumina Fluorescent spectrometer. Fluorescence lifetimes were obtained by Horiba DeltaFlex with 392 nm Laser NanoLED.

3. Methods

3.1 General testing method. The stock solutions of the **HF** or **HF-NO_2** were prepared in DMSO with concentration of 1 mM and restored in the refrigerator for further usage. For the test of spectroscopic properties, a volume of 20 μL of 1 mM probe stock solution was injected into 2 mL of PBS solution to yield detection solution with probe concentration of 10^{-5} M. After addition of analyte, the mixture was shaken for 10 seconds and then was measured by a fluorometer.

3.2 Preparation of liposome. According to the literature (Proc. Natl. Acad. Sci, 1996, 93, 11443-11447), L-α-phosphatidylcholine were dissolved in chloroform (0.1 M), and 20 μL of this solution was added into a 50-ml round-bottom flask containing 980 μL of chloroform and 100 μL of methanol. 7 mL of distilled water was then carefully added along the flask walls. The organic solvents was removed in a rotatry evaporator under reduced pressure, an opalescent fluid was obtained with a volume of approximately 5 mL. This solution was then diluted with distilled water into 40 mL, providing the working liposome solution (containing 50 μM of L-α-phosphatidylcholine).

3.3 Molecular Docking. The 3D geometry of the molecules was constructed using the Gaussian viewer, then optimized at the level of B3LYP/6-31g* with PCM implicit water solvent model. The ligand-free crystal structure of HSA (PDB ID: 4K2C) was taken from the Brookhaven Protein Data Bank (http://www.rcsb.org/pdb). The R-value and the resolution of the file were 0.213 and 3.23Å, respectively. Flexible ligand docking was performed by AutoDock 4.2 molecular docking program using the implemented empirical free energy function and the Lamarckian Genetic Algorithm. The Autogrid was used to calculate Grids. The grid spacing was 0.375 Å as default. 10 docking runs with 25,000,000 energy evaluations were performed. Based on the best conformation of the ligand, the selected bond of the best conformation was rotated -45°, -30°, -15°, 15°, 30°, 45°, and saved as pdb files respectively using GaussView 5.0. AutoDockTools was used for restricting the rotation of corresponding bonds, and the free binding energy was calculated following similar setting as above mentioned.

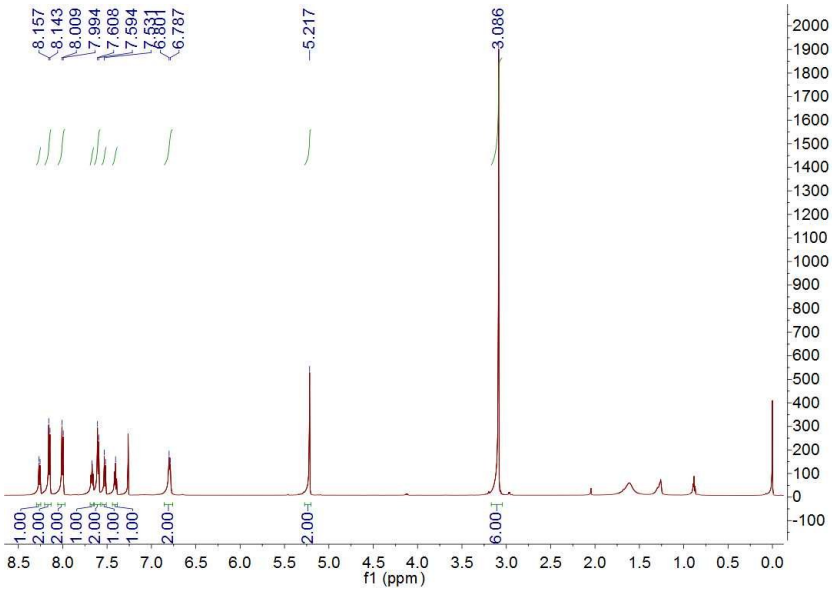
3.4 Displacement experiments. HSA/**HF-NO_2** complex was prepared by adding 20 μL of 1 mM **HF-NO_2** stock solution into 2 mL HSA (10 μM) solution, followed by shaking for 10 second. Then, two site-specific drugs warfarin (DS1) and ibuprofen (DS2) were added proportionally into HSA/**HF-NO_2** complex solution. After 1 minute mixing, the fluorescence intensity was measured at 490 nm.

3.5 Serum test. Five serum samples were kindly provided by Hematology Laboratory of Longhua District Central Hospital, Shenzhen. A volume of 20 μL of 1 mM probe stock

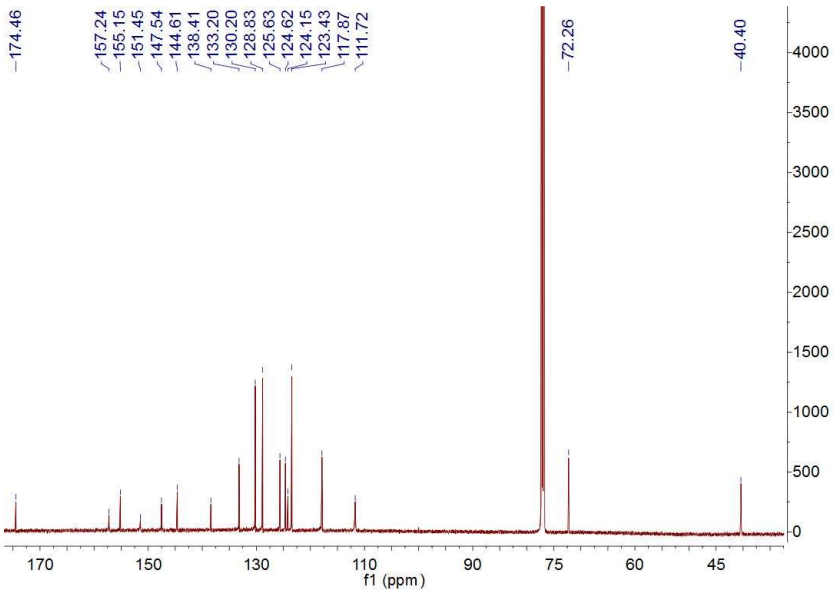
solution was firstly injected into 2 mL of PBS solution to yield detection solution with probe concentration of 10^{-5} M. Then serum sample was added into detection solution with different volumes. The final concentration of HSA ranging from 0.03 to 0.33 g/L was calculated based on the lab values by using BCG methods. Each serum sample was tested for 20 times by adding different volumes into detection solution, composing a total of 100 data points.

4. Synthesis and characterization

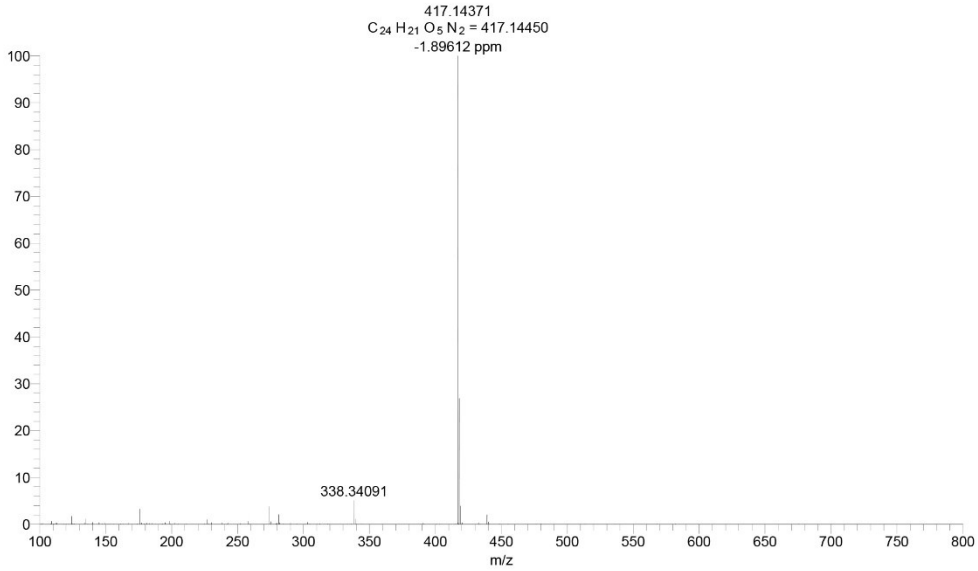
Synthesis of 2-(4-(dimethylamino)phenyl)-3-((4-nitrobenzyl)oxy)-4H-chromen-4-one **(HF-NO_2)**. **HF** (512 mg, 1.82 mmol) and 1-(chloromethyl)-4-nitrobenzene (468 mg, 2.73 mmol) were dissolved in 50 mL MeCN, followed by Cs_2CO_3 (889 mg, 2.73 mmol). The reaction mixture was heated to 60 ^oC and stirred for 12 h. After the solvent was removed under reduced pressure, the residue was purified by column chromatography on silica gel (Hexane/ethyl acetate = 5 /1), and the product was obtained as a yellow solid (546 mg, 72%). ^1H NMR (600 MHz, $CDCl_3$): δ = 8.27 (1H, d, *J* = 7.8 Hz), 8.16 (2H, d, *J* = 8.4 Hz), 8.01 (2H, d, *J* = 9 Hz), 7.67 (1H, m), 7.61 (2H, d, *J* = 8.4 Hz), 7.53 (1H, d, *J* = 8.4 Hz), 7.40 (1H, m), 6.80 (2H, d, *J* = 8.4 Hz), 5.22 (2H, s), 3.09 (6H, s). ^{13}C NMR (150 MHz, $CDCl_3$): 174.46, 157.24, 155.15, 151.45, 147.54, 144.61, 138.41, 133.20, 130.20, 128.83, 125.63, 124.62, 124.15, 123.43, 117.87, 111.72, 72.26, 40.40. HRMS: m/z calcd for $C_{24}H_{21}N_2O_5^+$ [M+H^+] 417.14450; found 417.14371.



^{1}H NMR spectrum of **HF-NO$_2$** recorded in $CDCl_3$.



^{13}C NMR spectrum of **HF-NO$_2$** recorded in $CDCl_3$.



HRMS of **HF-NO$_2$**

5. Additional Tables

Table S1 The information of simulated serum models

Simulated serum models	HSA	PL	CE	NEFA	TAG (g/L)
Healthy	35-55 (45)	1.11	0.74	0.15	1.56
FC hyperlipidaemia	35-55 (45)	2.07	1.20	0.22	5.05
Type 2 diabetes	35-55 (45)	1.40	0.87	0.21	2.49

Note: the average concentration of HSA was set as 45 g/L, and the data of blood lipids were cited from the literature (Progress in Lipid Research, 2008, 47, 348–380).

Table S2 The information of real serum samples

Sample No.	HSA (g/L)	Total TAG (mmol/L)	Total cholesterol (mmol/L)
1	23.9	0.60	1.77
2	44.2	1.76	4.39
3	52.8	2.09	3.30
4	46.7	2.01	3.69
5	39.9	3.15	3.41

6. Additional Figures

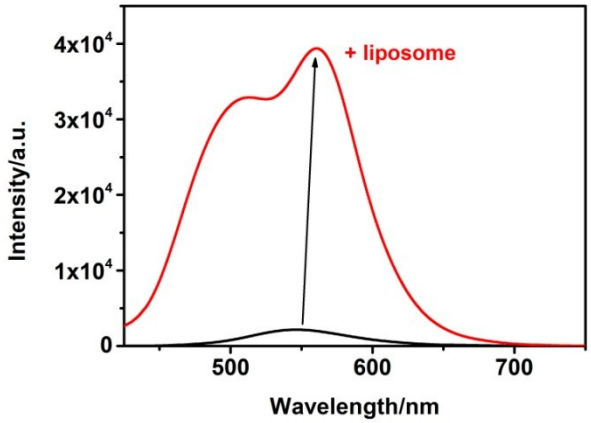


Figure S1. The fluorescence spectra of 10 μM **HF-NO$_2$** in liposome solution (containing 50 μM of L-α-phosphatidylcholine). λ_{ex} = 415 nm.

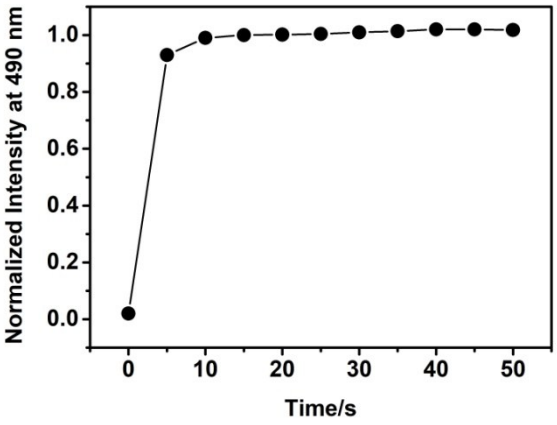


Figure S2. The time-dependent normalized intensity of 10 μM **HF-NO$_2$**. λ_{ex} = 415 nm.

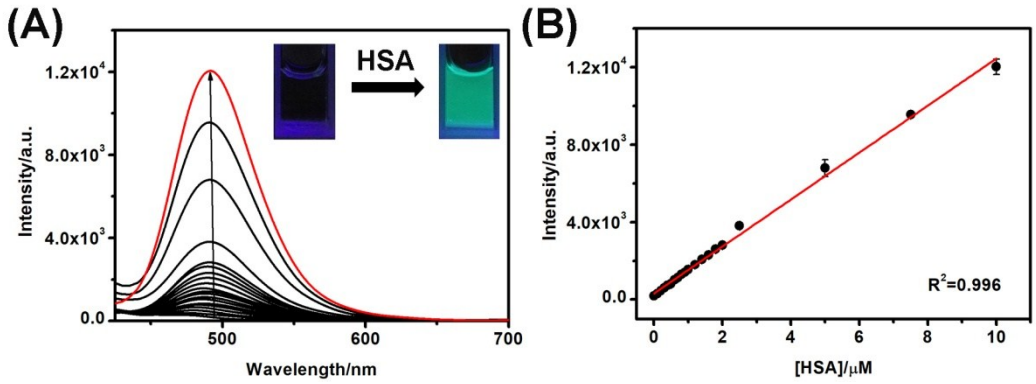


Figure S3. (A) The fluorescence spectra and (B) intensity changes (λ_{em} = 490 nm) of 10 μM **HF-NO$_2$** in PBS buffer upon addition of increasing concentration of HSA. λ_{ex} = 415 nm.

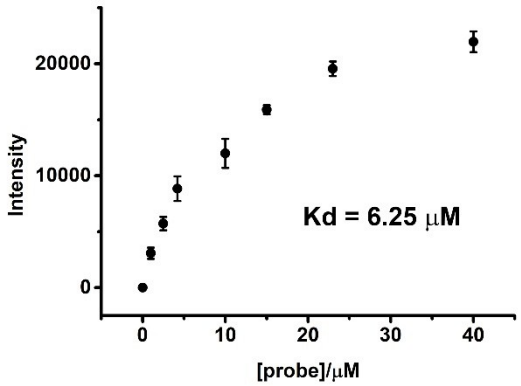


Figure S4. The fluorescence intensity changes (λ_{em} = 490 nm) of 10 μM HSA in PBS buffer upon addition of increasing concentration of **HF-NO$_2$**. λ_{ex} = 415 nm. The dissociation constant (K_d) was calculated based on following equation: $\Delta r/\Delta r_{max} = [L]/(K_d + [L])$, where Δr is the difference in fluorescence intensity in presence of the protein at concentration [L], and Δr_{max} is the maximum intensity change.

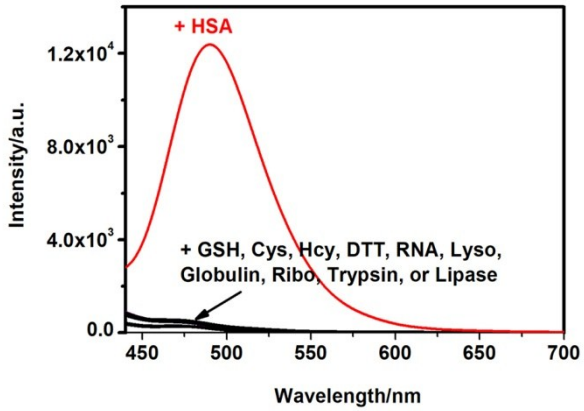


Figure S5. The fluorescence spectra of **HF-NO$_2$** in PBS buffer upon addition of 1 equivalent of HSA and other competitive components, including glutathione (GSH), cysteine (Cys), homocysteine (Hcy), dithiothreitol (DTT), ribonucleic acid (RNA), lysozyme (Lyso), globulin, ribonuclease (Ribo), trypsin, and lipase, respectively. [**HF-NO$_2$**] = [HSA] = [competitive compounents] = 10 μM. λ_{ex} = 415 nm

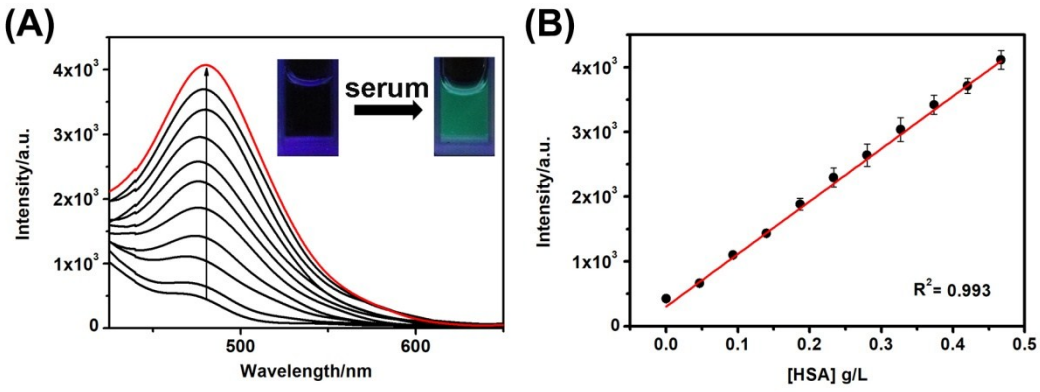


Figure S6. (A) The fluorescence spectra and (B) intensity changes (λ_{em} = 490 nm) of 10 μM **HF-NO$_2$** in PBS buffer upon titration of standard serum containing various concentration of HSA. λ_{ex} = 415 nm.

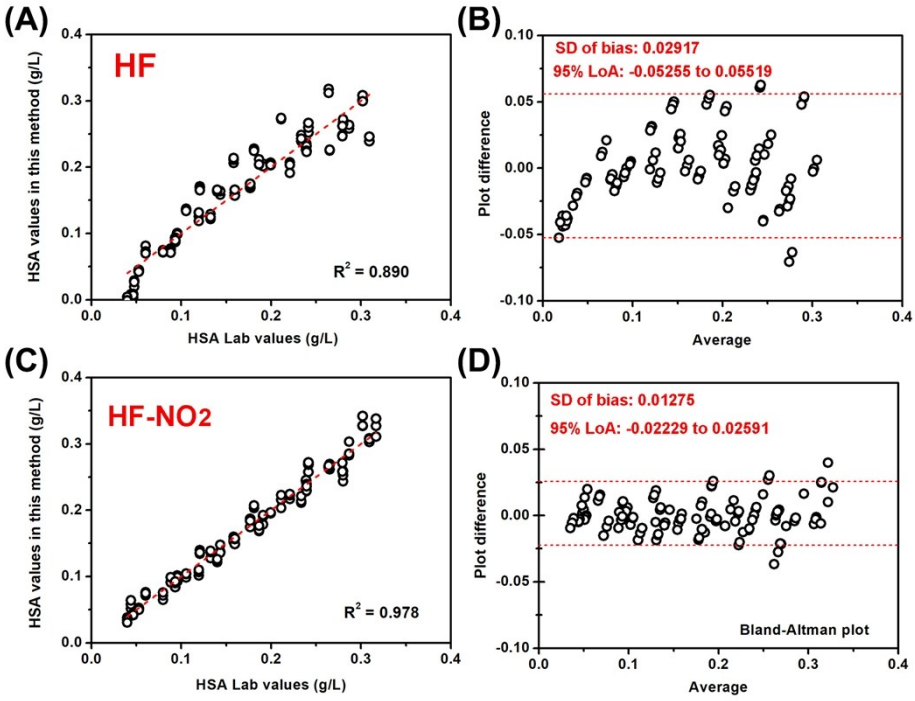


Figure S7. Comparison between linearity plots of HSA values by laboratory BCG method and HSA values measured by (A) **HF** and (C) **HF-NO$_2$**. Bland-Altman plots for HSA test by (B) **HF** and (D) **HF-NO$_2$** with the representation of standard deviation (SD) of bias and 95% limits of agreement (LOA).