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Brightness and stable dye-doped fluorescent polystyrene microspheres: quantitative lateral flow immunoassay detecting serum amyloid A

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Contents

1. General methods

Experimental materials and instruments

Unless special stated, all solvents and chemicals were purchased from commercial suppliers in analytical grade and used without further purification. Sodium dodecyl benzene sulfonate (SDS), Styrene, Potassium persulfate (KPS), Acrylic acid, Tetrahydrofuran (THF), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), N-Hydroxy succinimide (NHS), bovine serum albumin (BSA), boric acid, TRIS hydrochloride (Tris-HCl), Sodium tetraborate decahydrate, were purchased from Shanghai Titan Scientific Co., Ltd. PVC base, sample pad, nitrocellulose (NC) membrane, absorbent pad, were purchased from Shanghai Upper BIO-TECH PHARMA Co., Ltd. Serum amyloid A mAb1(SAA mAb1), serum amyloid A mAb2 (SAA mAb2), serum amyloid A antigen (SAA), Goat anti-mouse immunoglobulin (IgG), were purchased from Shanghai MEDIX Medical Technology Co., Ltd. An XYZ Large Platform Sensing Dispenser HM3260, Programmable Strip Cutter ZQ2002, and LM 4000 assay laminator (Biodot, Irvine, CA, USA) were used to prepare the detection strip. A portable LFIA strip reader was obtained from Shanghai Upper BIO-TECH PHARMA Co., Ltd (Shanghai, China). A high-speed freezing centrifuge (CR30NX) was obtained from Eppendorf (Hamburg, Germany). The Vortex-Genie2 was purchased from Scientific Industries (Bohemia, New York, USA). Nano-ZS90 Zeta Sizer (Malvern Panalytical Ltd, Shanghai, China) was used to characterize dynamic light scattering (DLS) and zeta potential.

Preparation of PS microspheres

Briefly, 1 mL of 0.5% SDS was added to 50 mL of deionized water, followed by the addition of 0.1 g of KPS, 5 mL of styrene, and 1 mL of acrylic acid. The reaction was then conducted for 6 hours at 500 rpm and a temperature of 75^{o}C. Subsequently, the mixture was filtered to obtain polystyrene (PS) microspheres.

Preparation of BFOH@PS

Briefly, 100 μL of 10 mg/mL PS microspheres were ultrasonically dispersed in 10 mL of 0.25% aqueous SDS solution. Subsequently, a THF solution containing 2 mg/mL of **BFOH** (1 mL) was added and heated to 60^{o}C for 1 hour to swell and dope. After allowing the solution to remain at temperature for evaporation THF for 1 hour, it was centrifuged at 20,000 rpm for 30 minutes and washed three times with water and ethanol, respectively, until the supernatant became non-fluorescent. Finally, the product was resuspended in 1 mL of water.

Preparation of immunofluorescent microspheres (BFOH@PS-SAA mAb2)

Initially, 50 μL **BFOH@PS** (5 mg/mL) was combined with 166.3 μL MES buffer (500 mM, pH 6.0) and vortexed for 1 minute. Then 15 μL of an aqueous solution of NHS and EDC (10 mg/mL) was added gradually to activate the carboxyl groups. The mixture was allowed to react for 30 minutes at room temperature, then centrifuged at 20,000 rpm for 30 minutes and the supernatant removed. The pellet was resuspended in MES buffer (500 mM, pH 7.0) by sonication and 29.4 μL of SAA mAb2 antibody (5.1 mg/mL) was added. The solution was then stirred for 1 hour before 100 μL of Tris-HCl buffer (500 mM, pH 8.0) and BSA (50 mg/mL) were added. The solution was then stirred for another hour. It was then centrifuged at 20,000 rpm for 30 minutes and the supernatant was discarded. The prepared BFOH@PS-SAA mAb2 was then resuspended in BB buffer (0.06 mM, pH 8.0) containing 0.9% NaCl and 0.1% sodium azide and stored at 4^{o}C for future use.

The LFIA strip by BFOH@PS-SAA mAb2

The LFIA strip consisted of four components: PVC plate, sample pad, NC membrane, and absorbent pad. SAA mAb1 antibody (1 mg/mL) and goat anti-mouse IgG (1 mg/mL) were applied to the NC membrane at a rate of 1 μL/cm for the T and C lines, respectively, spaced 4 mm apart, using an XYZ Large Platform Sensing Dispenser, and then dried for 24 hours at 37 ^{o}C. The lateral flow immunoassay (LFIA) strips were cut to a width of 3.8 mm using an automatic programmable strip cutter and stored in a plastic Ziplock bag.

Principle of portable LFIA strip reader

A 480 nm LED lamp was positioned at the top of the darkroom to provide a stable light source. The fluorescence signals passed through a 540 nm filter, were captured by a CMOS camera, and converted into digital signals. All signals were transmitted to a computer through a touchscreen interface.

Quantitative detection of SAA by LFIA strip

The standard procedure for SAA detection involved adding 3 μL of undiluted serum to 1 mL of BFOH@PS-SAA mAb2 solution and thorough mixing, Subsequently, 100 μL of this mixture was sampled, and fluorescence data was captured by a portable strip LFIA strip reader after 10 minutes. For quantitative analysis, an equation was formulated with the Log (Intensity of the T Line) on the vertical (y) axis and Log (Concentration of SAA antigen) on the horizontal (x) axis.

Absorption and fluorescence spectra

Determination the fluorescence spectrum and property of probes. Fluorescence and UV-Vis spectra of probes were recorded using a fluorescence spectrometer (Varian Cary Eclipse) and an ultraviolet-visible (UV-Vis) spectrophotometer (Varian Cary 500), respectively. UV-Vis spectra were acquired from 300 to 700 nm. The fluorescence measurement of **BFOH@PS**, **BFOH dyes** and **Rhodamine 6G** were carried out at an excitation wavelength of 480 nm and emission spectra scan in the range of 490-700 nm.

For spectra measurement, **BFOH@PS** (10 mg/mL) was diluted with 1X PBS (NaCl 8 g/L, KCl 0.2 g/L, Na_2HPO_4-$2H_2O$ 1.78 g/L, KH_2PO_4 0.27 g/L, pH 7.4) to a final concentration of 0.5 μg/mL, 1 μg/mL, and 2 μg/mL. BFOH stock solutions were prepared in THF at a concentration of 1 mM and stored at -20°C before use. **BFOH** stock solutions (1 mg/mL) was diluted with 1X PBS (NaCl 8 g/L, KCl 0.2 g/L, Na_2HPO_4-$2H_2O$ 1.78 g/L, KH_2PO_4 0.27 g/L, pH 7.4) to a final concentration of 0.25 μg/mL, 0.5 μg/mL, and 1 μg/mL. **Rhodamine 6G** stock solutions were prepared in DMSO at a concentration of 1 mM and stored at -20°C before use. Rhodamine 6G stock solutions (1 mM) was diluted with 1X PBS (NaCl 8 g/L, KCl 0.2 g/L, Na_2HPO_4-$2H_2O$ 1.78 g/L, KH_2PO_4 0.27 g/L, pH 7.4) to a final concentration of 1 μM, 2 μM, 3 μM.

The concentration-absorbance curve of BFOH in THF solution was established to quantitatively determine the concentration of doped dye in microspheres. The absorbance of BFOH solution (0.05-0.2 μg/mL, THF) at 480 nm was measured and plotted as Figure S15.

Measurement of quantum yield

Rhodamine 6G was used as standard for the measurements. The absorbance of each sample at 480 nm was recorded. Next, the fluorescence spectrum of each sample was measured with excitation at 480 nm (**BFOH@PS**: 490-700 nm, **BFOH**: 490-680 nm, **Rhodamine 6G**: 490-680 nm). The slope of integrated fluorescence intensity over absorbance was then calculated. Integrated fluorescence intensity was calculated using Prism (Area under Curve). The quantum yield was calculated using the following equation:

$$\Phi_{dye} = \Phi_{ST}(\frac{Slop_{dye}}{Slop_{ST}})$$

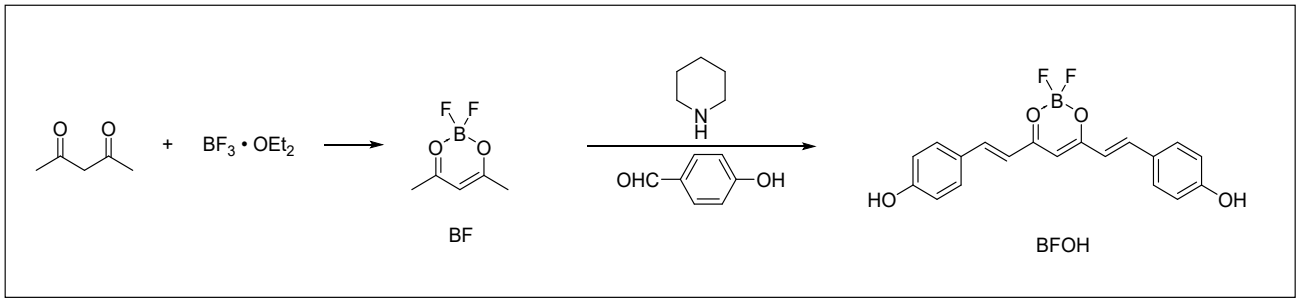
, where ϕ is quantum yield. The fluorescence quantum yield (QY) measurement of BFOH and BFOH@PS in PBS (10 mM, pH 7.4), the reference standard was rhodamine 6G in PBS with a QY of 95% [1]. The parameter of refractive index of the solvent is not shown in the equation because the same solvent was used for all the samples. All the measurements were performed at 25 °C.

The QY of BFOH@PS and BFOH was calculated to be 86.4% and 54.1% respectively.

Brightness calculation

To evaluate the performance of these dyes, it was essential to characterize their brightness (brightness = $\varepsilon_\lambda \times \Phi_F$), which is reliant on both absorption coefficient (ε_λ) and fluorescence quantum yield (Φ_F). From Figure S8, the brightness of BFOH@PS (epsilon ~ 32447 M^-1 cm^-1 and QY ~ 86.4%) is calculated as 28035; the brightness of BFOH (epsilon ~ 42602 M^-1 cm^-1 and QY ~ 54.1%) is calculated as 23047.

2. Syntheses and characterizations of BFOH



Scheme S1. Synthetic routes for **BF** and **BFOH**.

Synthesis of BF

In a dry 250 mL round-bottomed flask, acetylacetone (3.0 g, 30 mmol) was added, and boron trifluoride ethyl ether solution (4.3 g, 30 mmol) was slowly added dropwise under an ice bath, and the reaction was heated up to 60 °C with stirring and refluxing under the protection of argon for 4 h. After the reaction solution was cooled down to room temperature, 50 mL of petroleum ether was added, and the solid extracted, and then filtered. The crude product in the filter cake was washed by adding 10 mL of methanol, the filter cake changed from black to light yellow, and freeze-dried to get **BF** as a light yellow solid (4.0 g, 27.3 mmol): Yield 91.3%. ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 2.33 (s, 6H, -CH_3), 5.99 (s, 1H, -CH-). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 24.26, 101.79, 192.43 Mass spectrometry (ESI-MS, m/z): $[M+H]^+$ Calcd. for $C_5H_7BF_2O_2$: 149.0580; found: 149.0230.

Synthesis of BFOH

BF (500 mg, 3.4 mmol) and 4-hydroxybenzaldehyde (854 mg, 7.0 mmol) were dissolved in acetonitrile (20 mL) with piperidine (0.5 mL). The mixture was then heated to 60 °C under an argon atmosphere and refluxed for 4 hours. The solvent was removed under reduced pressure, and then the crude product was purified by silica gel chromatography using petroleum ether/ethyl acetate (v/v, 5:1) as eluent to afford **BFOH** as a dark red solid (1.05 g, 2.9 mmol): Yield 87%. ^{1}H NMR (400 MHz, DMSO-d_6, ppm): δ 6.46 (s, 1H, -CH-), 6.87 (d, J = 8.4 Hz, 4H, Ph-H), 6.97 (d, J = 15.6 Hz, 2H, Vinyl-H), 7.74 (d, J = 8.8 Hz, 4H, Ph-H), 7.92 (d, J = 15.6 Hz, 2H, Vinyl-H), 10.46 (s, 2H, -OH). ^{13}C NMR (100 MHz, DMSO-d_6, ppm): δ 101.26, 116.28, 117.62, 125.43, 131.98, 146.64, 161.57, 178.79. Mass spectrometry (ESI-MS, m/z): $[M-H]^-$ Calcd. for $C_{19}H_{14}O_4BF_2$: 355.0948; found: 355.0965.

3. Supporting figures

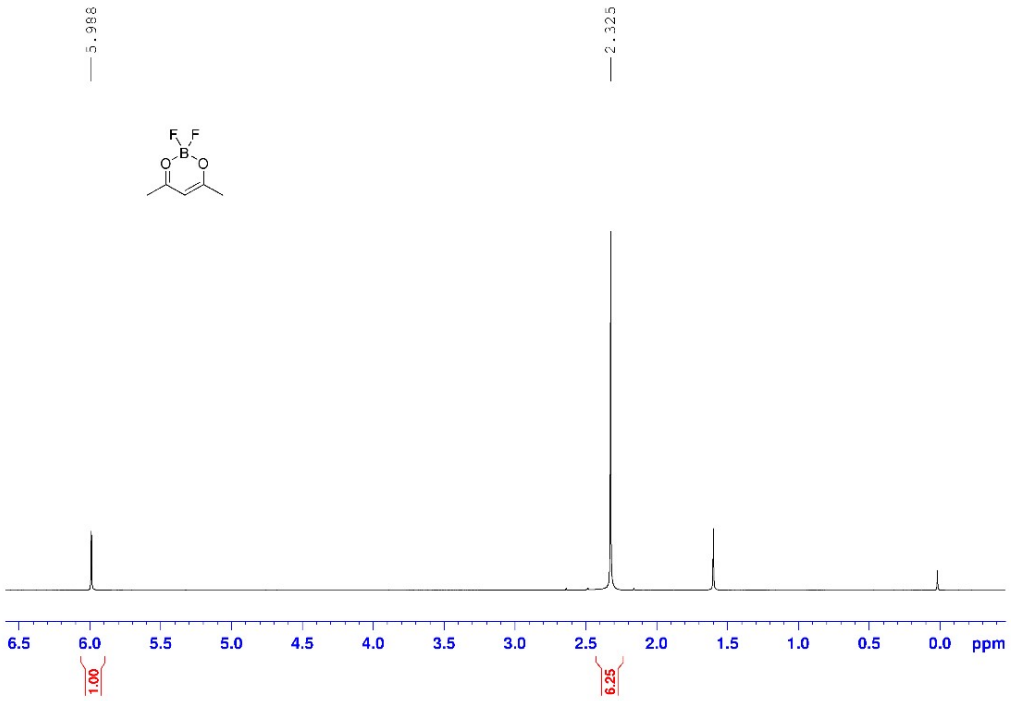


Figure S1. ^{1}H NMR spectrum of **BF** in $CDCl_3$

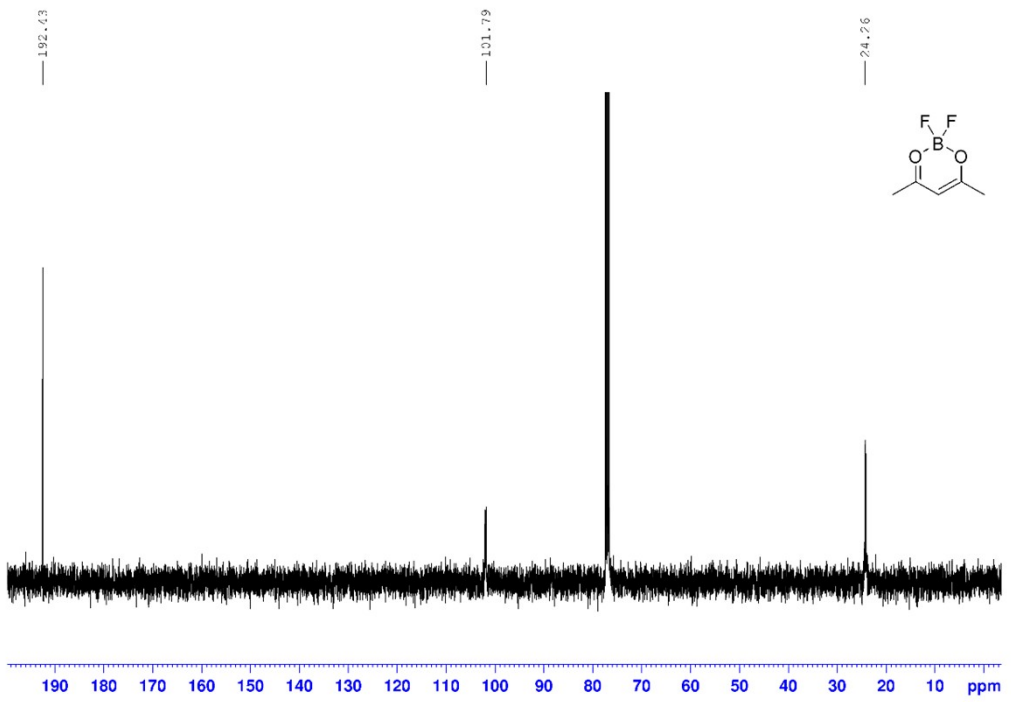


Figure S2. ^{13}C NMR spectrum of **BF** in $CDCl_3$

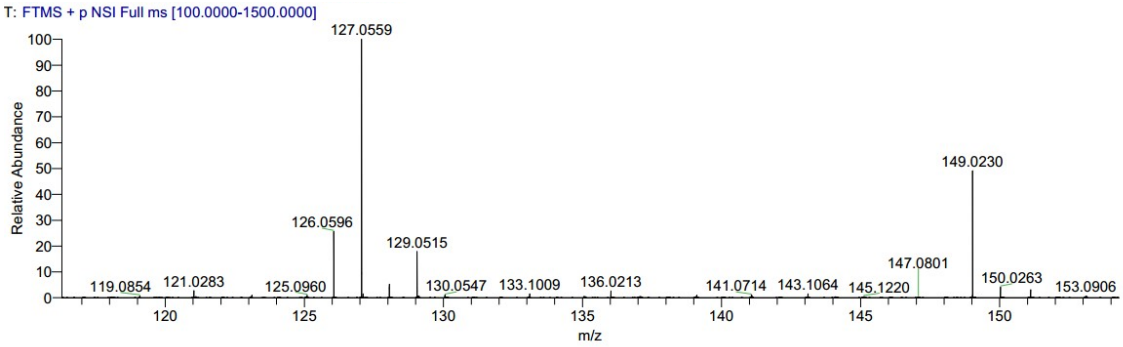


Figure S3. HRMS spectrum of **BF**

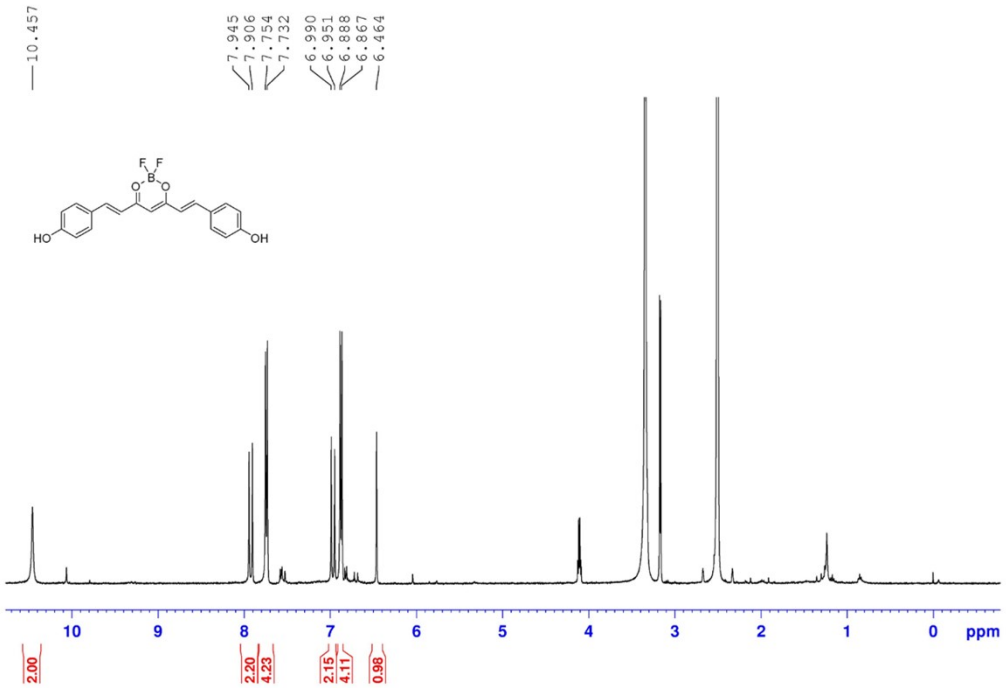


Figure S4. ^{1}H NMR spectrum of **BFOH** in DMSO-d_6

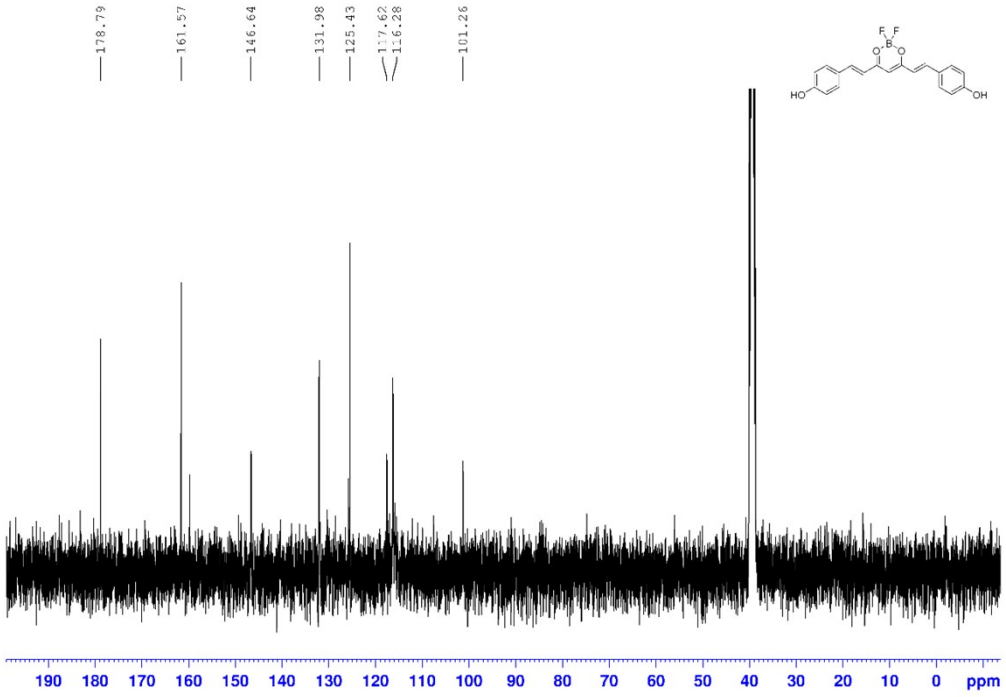


Figure S5. ^{13}C NMR spectrum of **BFOH** in DMSO-d_6

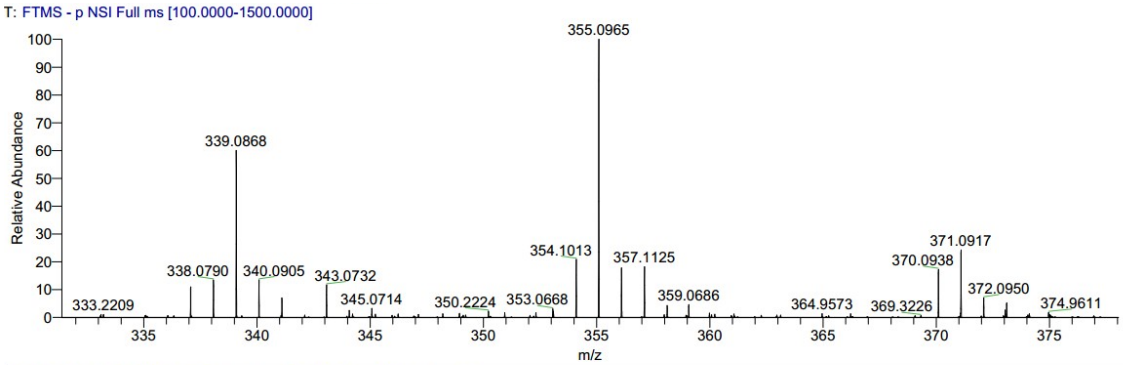


Figure S6. HRMS spectrum of **BFOH**

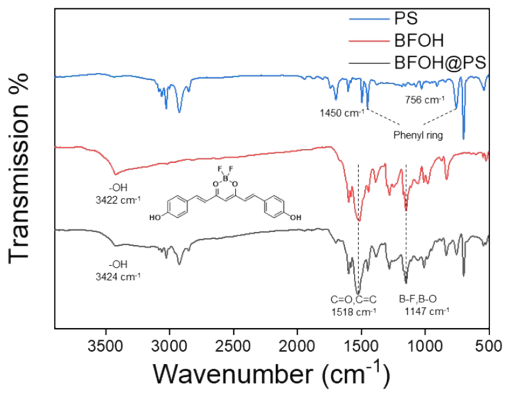


Figure S7. The FTIR spectrum of PS (blue line), BFOH (red line), and BFOH@PS (black line).

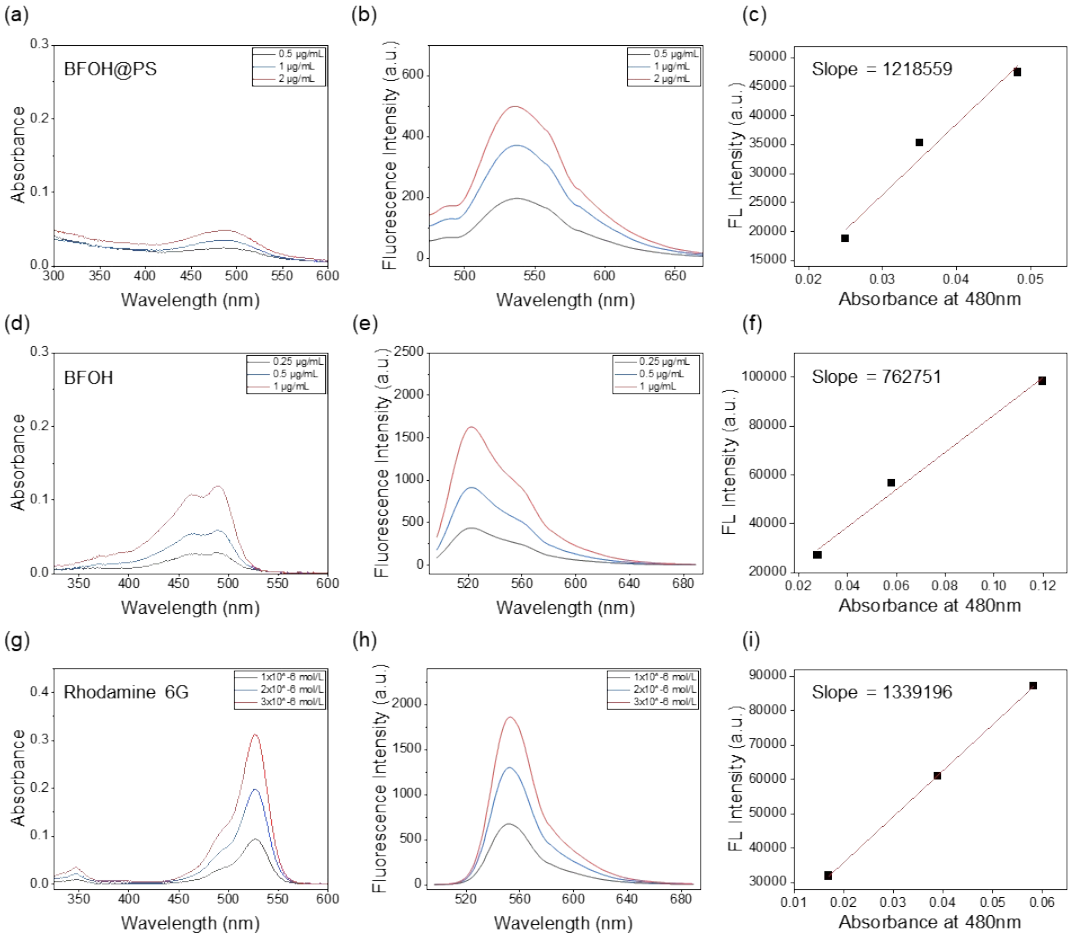


Figure S8. The fluorescence quantum yield (QY) measurement of BFOH and BFOH@PS in PBS (10 mM, pH 7.4), the reference standard was rhodamine 6G in PBS with a QY of 95% [1]. (a) UV-vis absorption spectra of BFOH@PS in PBS with increasing concentration. (b) Fluorescence spectra of BFOH@PS in PBS with increasing concentration. (c) Integrated fluorescent intensity plotted as a function of optical density (OD) at 480 nm for BFOH@PS based on the measurements in a) and b). (d) UV-vis absorption spectra of BFOH@PS in PBS with increasing concentration. (e) Fluorescence spectra of BFOH in PBS with increasing concentration. (f) Integrated fluorescent intensity plotted as a function of optical density (OD) at 480 nm for BFOH based on the measurements in d) and e). (g) UV-vis absorption spectra of Rhodamine 6G in PBS with increasing concentration. (h) Fluorescence spectra of Rhodamine 6G in PBS with increasing concentration. (i) Integrated fluorescent intensity plotted as a function of optical density (OD) at 480 nm for Rhodamine 6G based on the measurements in g) and h). The QY of BFOH@PS and BFOH was calculated to be 86.4% and 54.1% respectively. All fluorescence spectra were conducted under consistent laser power levels at 480 nm laser excitation.

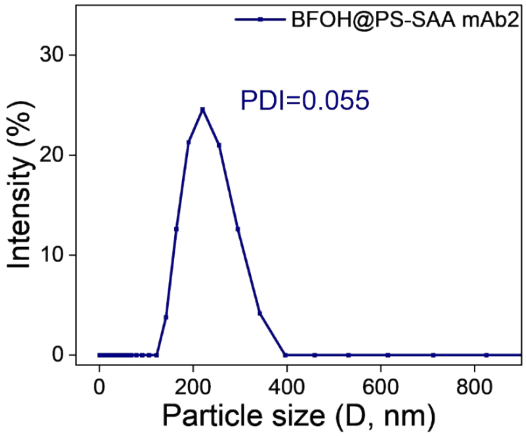


Figure S9. Hydrodynamic size of BFOH@PS-SAA mAb2.

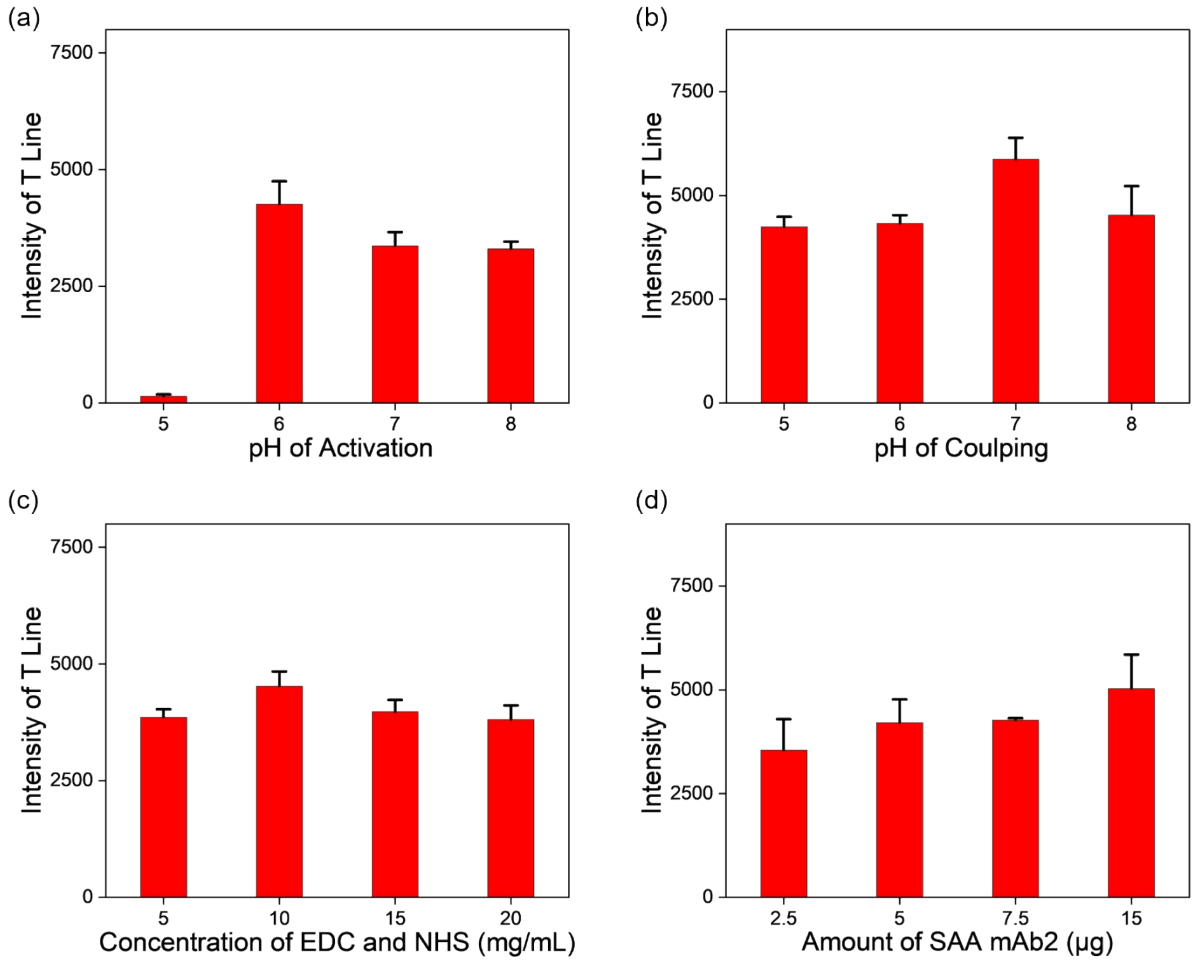


Figure S10. Optimization of parameters about BFOH@PS-SAA mAb2 LFIA strip performance. (a) Intensity of T Line under different pH of activation. (b) Intensity of T Line under different pH of coupling. (c) Intensity of T Line under different Concentration of EDC and NHS. (d) Intensity of T Line under different amount of SAA mAb2. The concentration of the SAA is 50 mg/L, respectively. Data with error bars are expressed as mean ± s.d., n = 3.

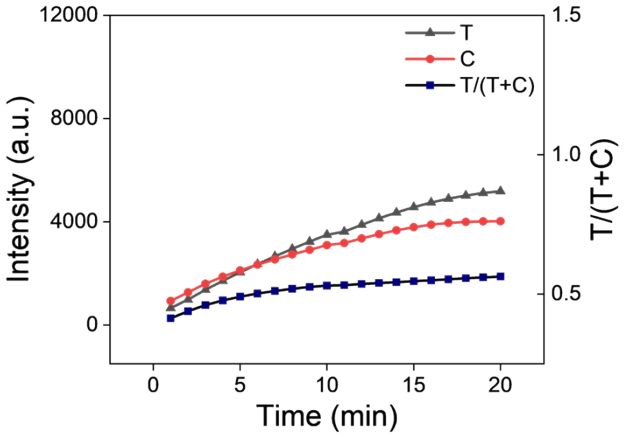


Figure S11. Fluorescent intensity of the T and C lines with temporal evolution

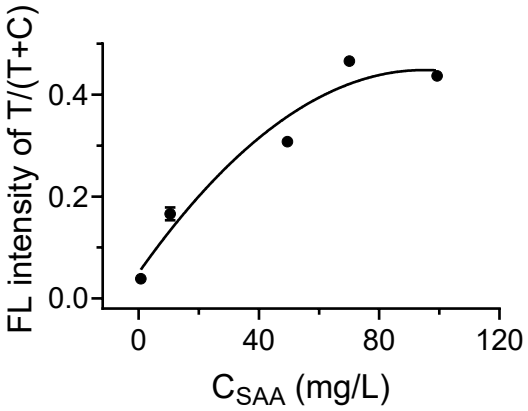


Figure S12. Standard curve of T/(T+C) ratio and SAA concentration.

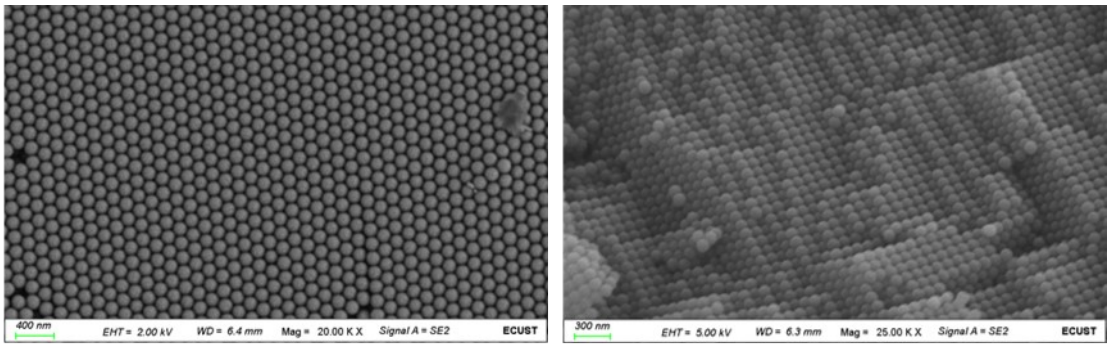


Figure S13. The FESEM micrographs of the dye-doped microspheres from different batches.

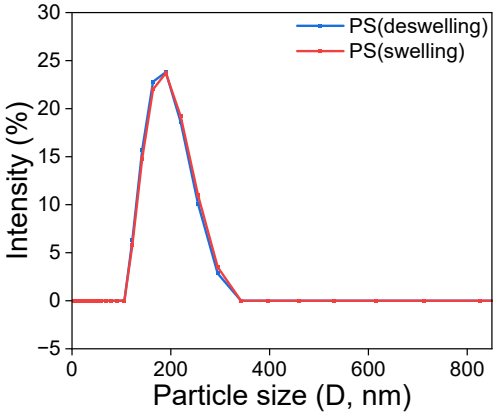


Figure S14. The hydrodynamic size of PS swelling/deswelling experiment in the absence of dye.

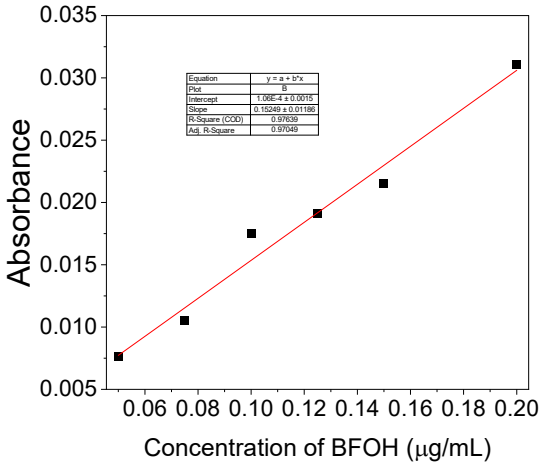


Figure S15. Concentration-dependent absorption standard curve for the BFOH dye in THF.

Table S1 Buffer for lateral flow assay.

Reagent name	Usage amount
$Na_2HPO_4 \cdot 12H_2O$	4.9 g
$NaH_2PO_4 \cdot 2H_2O$	1.298 g
NaCl	5 g
BSA	10 g
Glucose	1 g
Sucrose	1 g
Tween-20	1 mL
Proclin 300	1 mL
50 mg/mL Gentamicin sulphate	1 mL
Purified water	1000 mL

Table S2 Boric acid-borax buffer solution formulation.

Reagent name	Usage amount
Boric acid	0.2 g
Borax	0.17 g
Purified water	100 mL

Table S3 Clinical sample information.

		Number	Date	Age	Gender	SAA concentration (mg/L)
1	2023-05-04	32	Male	3.84		
2	2023-05-04	37	Male	4.14		
3	2023-05-08	63	Female	4.21		
4	2023-05-09	72	Female	4.36		
5	2023-05-08	30	Female	4.79		
6	2023-05-04	44	Male	5		
7	2023-05-04	84	Male	5.06		
8	2023-05-04	31	Female	10.16		
9	2023-05-04	44	Female	8.61		
10	2023-05-05	63	Male	7.79		
11	2023-05-04	31	Female	6.19		
12	2023-05-05	49	Female	5.41		
13	2023-05-06	41	Male	5.21		
14	2023-05-09	72	Female	13.19		
15	2023-05-07	67	Female	19.8		
16	2023-05-04	54	Female	21.46		
17	2023-05-05	63	Male	42.05		
18	2023-05-05	66	Male	44.84		
19	2023-05-04	40	Male	65.13		
20	2023-05-05	79	Female	69.19		
21	2023-05-06	29	Male	84.89		
22	2023-05-04	88	Female	39.87		
23	2023-05-04	33	Female	42.32		
24	2023-05-04	80	Male	50.35		
25	2023-05-08	67	Male	73.08		

4. Reference

[1] A. M. Brouwer, *Pure Appl. Chem.*, **2011**, *83*, 2213-2228.