Time-Insensitive Fluorescent Sensor for Human Serum Albumin and Its Unusual Red Shift

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Supporting Information
Materials
Human serum standard (ERM-DA470k/IFCC) was purchased from European Reference Materials. Bromocresol green, potassium phosphate monobasic, tris(hydroxymethyl) aminomethane (Tris), 4-(2-hydroxymethyl)piperazine-1-ethanesulfonic acid (HEPES), succinic acid, Brij-35 solution, 2’,7’-dichlorofluorescein, and dimethyl sulfoxide were purchased from Fisher Scientific (Pittsburgh, PA). Purified water was prepared using a MilliQ filtration system. Fluorescein derivatives; Pittsburgh Green, Pittsburgh Green II, Pittsburgh Green III, Pittsburgh Yellow Green, and Pittsburgh Yellow Green II, were prepared by synthesis.

Instrumentation
All analysis was performed using a Modulus II, microplate multimode reader. Fluorescence measurements were obtained using a blue excitation filter (excitation wavelength: 490 nm, emission wavelength range: 510–570 nm). Absorbance with bromocresol was measured at 650 nm.

Quenching Effect of Human Serum Albumin on Fluorescein Derivatives
2’,7’-Dichlorofluorescein and five fluorescein derivatives; Pittsburgh Green, Pittsburgh Green II, Pittsburgh Green III, Pittsburgh Yellow Green, and Pittsburgh Yellow Green II, were examined. A 2.00 g/L stock solution of HSA in a 50 mM phosphate pH 7.0 buffer was combined with a 4 µM solution of each fluorescent compound. Final concentrations within samples were 1 g/L HSA, 2 µM fluorescein derivative, and 0.2% DMSO in a 50 mM phosphate pH 7.0 buffer. Controls were prepared without HSA. A mixture containing no fluorescein derivative served as a negative control. Five replicates were performed for all samples.

Effect of pH 7 Buffers on Fluorescence Quenching
Samples were prepared using 50 mM phosphate, 50 mM Tris, or 50 mM HEPES buffers. Final concentrations were 1 g/L HSA, 2 µM Pittsburgh Green II, and 0.2% DMSO in the corresponding pH 7.0 buffer. HSA was excluded from all control mixtures. Five replicates were performed for all samples.

Preparation of Pittsburgh Green II Standard Curve
Standards were prepared by diluting from a 2 g/L HSA stock solution in a 50 mM HEPES pH 7.0 buffer. The final concentration of Pittsburgh Green II was 2 µM with 0.2% DMSO. A 23 point standard curve was prepared with a HSA concentration range of 0–50 mg/L. Five replicates were performed for all samples.

Preparation of Bromocresol Green Standard Curve
A solution of bromocresol green was prepared as previously described. In brief, a 0.60 mM solution of bromocresol green was prepared in 1 mM NaOH in water. The working solution consisted of one volume bromocresol green solution, three volumes 0.10 M succinate buffer, and 4 mL 30% Brij-35 solution. The pH was adjusted to 4.20.

Standards were prepared by diluting from a 2 g/L HSA stock solution in a 50 mM HEPES pH 7.0 buffer. The final concentration of bromocresol green was 75 µM, with 37.5 µM succinate buffer, and 0.2% DMSO. Absorbance was read at 650 nm upon the addition of the bromocresol green working solution. The standard curve was prepared with a HSA concentration range of 9–350 mg/L. All samples were performed in triplicate.

Analysis of European Standard Human Serum (ERM-DA470k/IFCC)
The dried albumin standard was reconstituted in MilliQ water (1.000 mL). The HSA concentration was corrected for the reconstitution mass by multiplying the certified concentration (37.2 g/L) by the ratio of the intended mass to the reconstituted mass. The corrected HSA concentration was determined to be 36.9 g/L.
For analysis by the bromocresol green method, the standard serum was diluted 100-fold in a 50 mM HEPES pH 7.0 buffer. This dilution was combined with the bromocresol green working solution to give a final concentration of 75 µM bromocresol green and 37.5 µM succinate buffer with 0.2% DMSO. The absorbance at 650 nm was read upon the addition of the dye. The concentration of the serum standard was determined by comparing to the calibration curve. Two trials were performed for the standard, in triplicate.

For analysis by the Pittsburgh Green II method, the serum standard was diluted 2000-fold in a 50 mM HEPES pH 7.0 buffer. This dilution was combined with Pittsburgh Green II, resulting in a final concentration of 2 µM with 0.2% DMSO. The concentration of the serum standard was determined by comparing to the standard curve. Two trials were performed with five replicates.

**Time-dependence of HSA Detection by Pittsburgh Green II**

A solution of Pittsburgh Green II (2 µM) and HSA (7.5 mg/L) and a solution of Pittsburgh Green II (2 µM), both in a 50 mM HEPES pH 7.0 buffer, were transferred to a 96-well plate, and the fluorescence signals were monitored for 30 min.

**Visualization of HSA (Figure 4a)**

In two 4-mL glass vials, 10-mM Pittsburgh Green II solution in DMSO (100 µL each) was transferred. 1 g/L HSA in Ultrapure water (125 µL) was transferred to one vial immediately. Both vials were diluted with Ultrapure water to a volume of 1.0 mL and incubated at 25 °C for 10 min. A 50 mM HEPES pH 7 buffer (1.0 mL) was added to each vial, and the resulting solutions were incubated at 25 °C for 5 min before a photograph of the solutions was taken under ambient light.

**Titration of Pittsburgh Green II with Normal Human Serum for Absorption Spectra (Figure 4b)**

Normal human serum (0–20 µL) was added to 80 µM Pittsburgh Green II in a 50 mM HEPES pH 7 buffer containing 0.8% DMSO (1.00 mL). Immediately after addition, the solutions were vigorously mixed, and the absorption spectra acquired.

**Titration of Pittsburgh Green II with Normal Human Serum for Emission Spectra (Figure 4c)**

A 10 mM Pittsburgh Green II solution (160 µL) in DMSO was diluted with 50 mM HEPES pH 7 buffer (19.84 mL) to yield an 80 µM Pittsburgh Green II solution in 0.8% DMSO/buffer. Normal human serum (0–50 µL) was added to the 80 µM Pittsburgh Green II solution (1.00 mL) at 25 °C. The resultant solution was vigorously mixed, and emission spectra acquired immediately. The samples were excited at 470 nm.

**Absorption spectra of fluorescein derivatives under different pHs (Figures 4d and 4e)**

To an 80 µM solution of Pittsburgh Green II in a 50 mM HEPES pH 7 buffer and 0.8% DMSO, 37% hydrochloric acid, then 3% hydrochloric acid in Ultrapure water, was added dropwise until at pH 7.05, 5.38, 5.10, and 3.98. The absorption of the solution at each pH was measured immediately.

To an 80 µM solution of 2’,7’-dichlorofluorescein in a 50 mM HEPES pH 7 buffer and 0.8% DMSO, 37% hydrochloric acid, then 3% hydrochloric acid in Ultrapure water, was added dropwise until at pH 7.01, 4.67, and 3.53. The absorption of the solution at each pH was measured immediately.