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Technical Note

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

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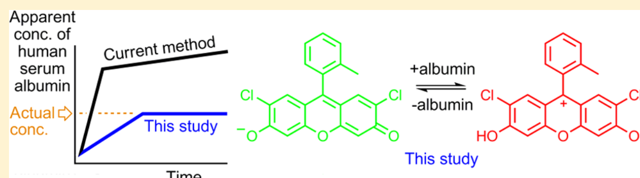
S *Supporting Information*

ABSTRACT: The concentration of human serum albumin (HSA) indicates the health state of individuals and is routinely measured by UV spectroscopy with bromocresol. However, this method tends to overestimate HSA, and more critically, depends highly on the timing, in seconds, of the measurements. Here, we report an analog of 2′,7′-dichlorofluorescein that can be used as a fluorescent sensor to quantify HSA in human sera. The accuracy of this new method proved superior to that of bromocresol when an international standard serum sample was analyzed. This method is more convenient than the bromocresol method because it allows for fluorescence measurements during a >15 min period. Colorimetric analysis was also performed to further investigate the effects of the binding of the sensor to HSA. These spectroscopic studies suggest that absorption and emission changes upon HSA binding may be due to the dehydration of the dye and/or stabilization of the tritylic cation species.



Albumin is a large globular protein found in large abundance in human sera.[1] It serves many functions, including the binding of exogenous species, which may provide a detoxification mechanism.[1,2] Quantification of albumin is clinically important, as it has been found to be an effective prognostic indicator. Human serum albumin (HSA) levels are typically ∼50 g/L (∼0.75 mM) for healthy individuals. A significant decrease in serum albumin is currently used in the staging system for multiple myeloma,[3] mortality,[4] and cognitive impairment prediction in the elderly[5] and is also used as an indicator for coronary heart disease.[6] Extremely low HSA concentrations (below 1 g/L) define analbuminemia, a rare congenital disorder.[7] Thus quantifying HSA, especially in low concentrations, is of great significance.

HSA is currently measured using colorimetric techniques such as bromocresol[8] green (BCG)[9−11] or bromocresol purple.[12−14] Among the shortcomings of the bromocresol method is the overestimation of albumin, especially in low concentrations.[15−18] This is alarming because the overestimation of albumin overlooks the aforementioned illness and poor prognosis. Even more significantly, the bromocresol method is highly time-sensitive; albumin concentrations are overestimated after 30 s of incubation because the dye begins to react with other proteins.[19,20] The time sensitivity poses a technical problem, rendering it difficult to develop a bromocresol-based high throughput assay to measure albumin in parallel. Additionally, at-home kits that would be available to the general public cannot be easily developed. New methods to measure serum proteins continue to be reported in recent years,[21,22] but the time sensitivity of the bromocresol-based method has not yet been resolved.

Here, we present a new fluorescence method that allows for the quantitative measurement of HSA. The on−off fluorescent sensor can readily be available via a one-step chemical synthesis.[23] The assay protocol is simple, with a fluorometer or fluorescence plate reader as the only instrument. The method is not time sensitive, an advantage for potential high throughput screenings and reproducibility.

We previously reported a fluorescence method to detect ozone in biofluid samples.[24] In this method, the fluorescent reporter was the 2′,7′-dichlorofluorescein (DCF) derivative **1** (Figure 1). In this previous work, we successfully demonstrated that the method could detect a high concentration of ozone with compound **1** as the reporter in a tissue culture media.[24] However, it was later found that a minute amount of ozone could not be detected in serum (data not shown). We hypothesized that compound **1** might be bound to biomacromolecule(s) in the tissue culture media and that its fluorescence was quenched by forming the spirocyclic form of compound **1**. To test this hypothesis, the fluorescence of compound **1** was measured in serum-containing pH 7 phosphate buffer and found to be quenched (data not shown). After removing the total proteins from the serum, the quenching effect was absent (data not shown). It was concluded that serum protein(s) were responsible for the quenching.

Albumin is the most abundant protein in serum, prompting us to hypothesize that albumin caused the fluorescence quenching. To test this hypothesis, the fluorescence of **1** was measured in the presence of HSA in a pH 7 phosphate buffer. As Figure 2a shows, the fluorescence signal decreased in the presence of HSA. In order to probe the structure-dependence of the fluorescence quenching, we tested closely related

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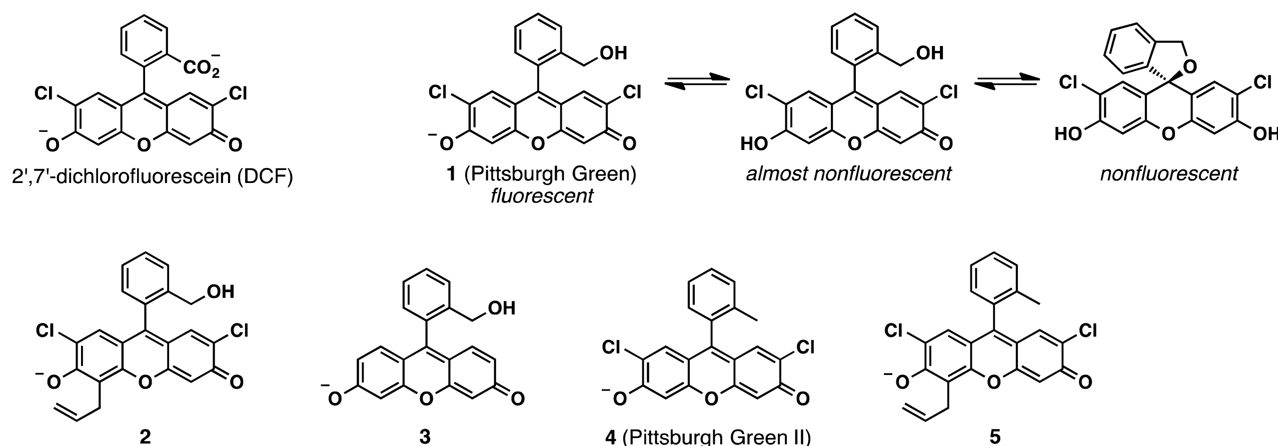

Figure 1. Structures of fluorescein derivatives used in this study.

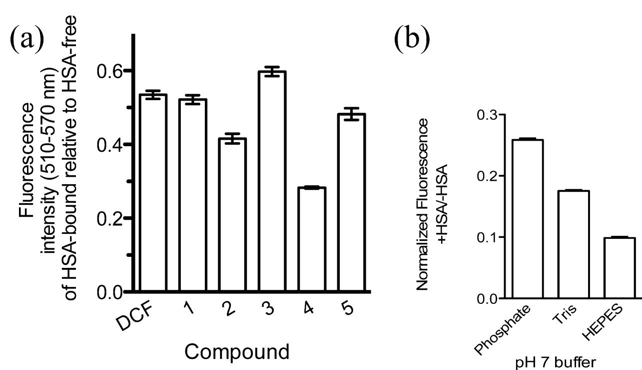

Figure 2. (a) Quenching effect of HSA on fluorescein derivatives (triplicate). (b) Effect of pH 7 buffer components on HSA detection with Pittsburgh Green II (triplicate).

compounds. The fluorescence of DCF was also quenched by HSA to a similar extent. Compound **2**[25] was quenched by HSA by a slightly larger degree. In order to determine the effect of the chloride groups of **1**, the previously prepared nonchloro fluorescein derivative **3**[26] was tested and found to be less responsive than the corresponding dichlorinated derivative **1**. This result indicates the importance of the chlorides for HSA binding.

At this stage, we hypothesized that DCF and compounds **1**, **2**, and **3** could be quenched by protonation on the phenolic hydroxy group or spirocyclization (Figure 1, equilibrium for **1**). Compound **4**[23] cannot form a spirocyclic structure and, thus, was considered a desirable probe for use in studying the effect of the spirocyclization. We found that this compound was quenched by HSA more prominently than **1**. This result excludes the spirocyclization as a fluorescence quenching mechanism and indicates that the methyl group is preferable over the hydroxymethyl group on the upper phenyl ring for HSA binding. Compound **5** was less responsive than **4** to HSA. From these studies, compound **4** (Pittsburgh Green II) emerged as an optimal fluorescent sensor for HSA.

Thus far, all fluorescence measurements were carried out in a phosphate pH 7 buffer, as this buffer was used to study fluorescein-HSA binding in the past.[21,27] We asked whether phosphate buffers were optimal for HSA-Pittsburgh Green II binding. To answer this question, we screened three commonly used pH 7 buffers (Figure 2b). Pittsburgh Green II was more responsive to HSA in a pH 7 HEPES buffer than in pH 7 phosphate or Tris buffers. Thus, the rest of this study was performed in a pH 7 HEPES buffer.

Next, we set out to determine whether Pittsburgh Green II could be used as a quantitative fluorescent sensor for HSA. As Figure 3a shows, the green fluorescence signal correlated with the concentration of HSA in a pH 7 HEPES buffer. A linear relationship was observed over a concentration range of 2−10 mg/L. This range is far lower than that of the traditional bromocresol method (100−300 mg/L),[28] indicating superior sensitivity.

We critically pondered whether the fluorescence change could quantify HSA in a time-insensitive manner. The green fluorescence signal was remarkably stable for at least 16 min in the presence of HSA (Figure 3, panels b and c). It should be noted that the signal initially decreased for the first 10 min without HSA when measured every minute, after which the background signal was stable. The initial background signal decrease was caused by photobleaching during the frequent excitation of the fluorophore, as indicated in Figure 3d. When HSA-containing samples were monitored over 30 min, the apparent concentration of HSA, as determined by the difference between fluorescence signals with and without HSA at each time point, changed as shown in Figure 3c. Notably, there is a substantial period of time (between 14 and 30 min) during which the apparent HSA concentration was essentially unchanged. In contrast, the apparent concentration of HSA using the BCG method rapidly changed around the recommended time (30 s). The study on the BCG method by Gustafsson showed that this widely used method could overestimate the HSA concentration by more than 12% in the first 90 s.[20] Our method provides a greater range of time points to measure HSA concentrations accurately, allowing the preparation and measurement of many samples in parallel.

In order to assess the accuracy of the Pittsburgh Green II-based method for quantifying HSA in human serum, we compared this method with the BCG method by using a certified reference material human serum standard (ERM-DA470k/IFCC) whose HSA concentration was determined to be 36.9 ± 1.2 g/L as the average of many analytical methods.[29] This sample was diluted by 200 fold for the BCG method and by 4000 fold for our method; because our method is 1 order of magnitude more sensitive than the BCG method, we diluted the samples accordingly to adjust the concentration of HSA to the linear range. The BCG method and our method indicated that the HSA concentration was 43.8 ± 1.3 and 36.5 ± 1.7 g/L,

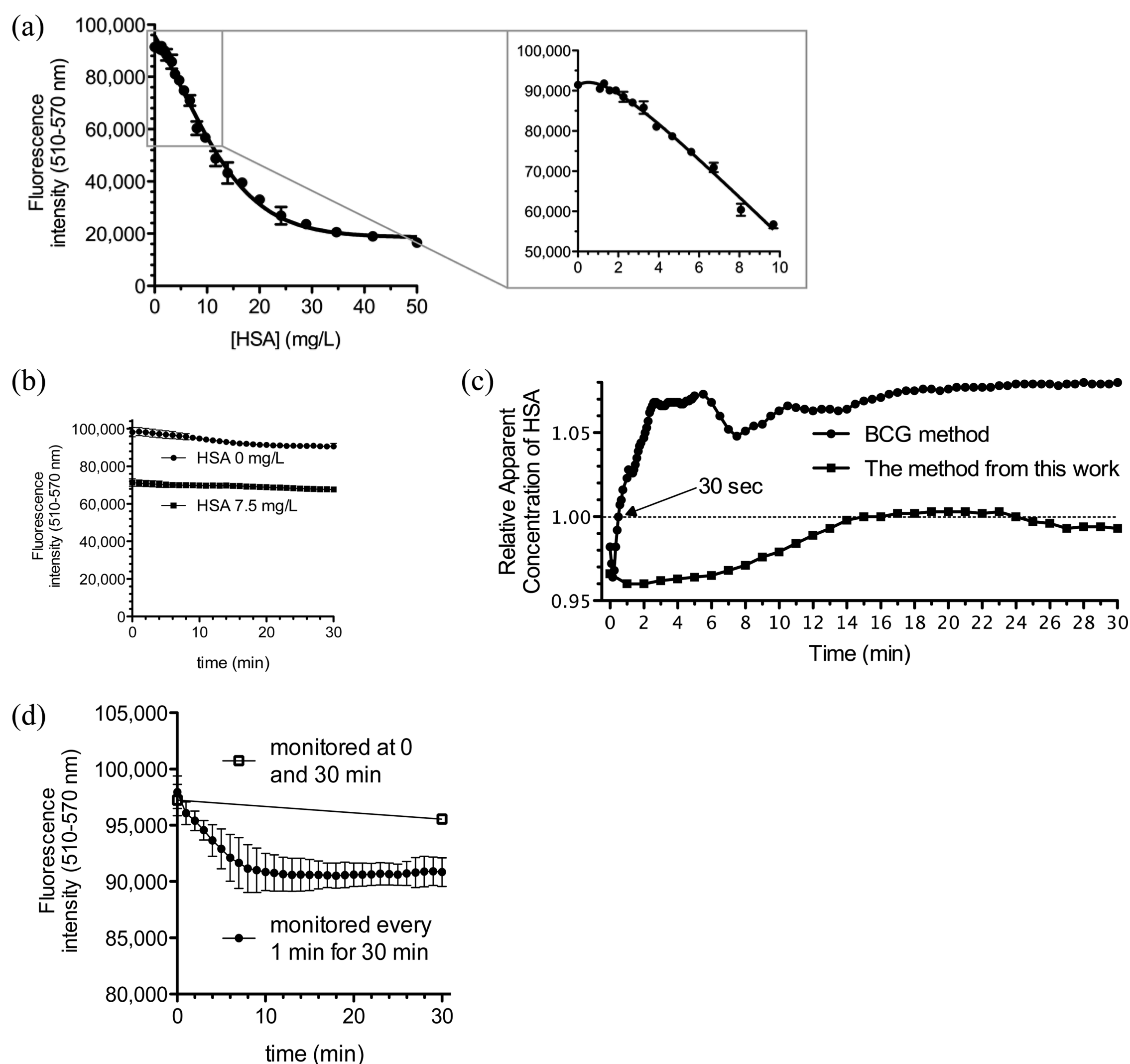

Figure 3. [Pittsburgh Green II] = 2 μM for all of the graphs. (a) Standard curve with Pittsburgh Green II (triplicate). (b) Time-dependence of HSA detection method with Pittsburgh Green II. (c) Time-dependence of HSA detection method with bromocresol green (BCG) and our method. The data were normalized. (d) Pittsburgh Green II can be photobleached when frequently monitored.

respectively, by one coauthor, and was 44.9 ± 3.6 and 38.9 ± 2.7 g/L, respectively, by another coauthor. In both cases, the bromocresol green method resulted in a significant difference from the accepted value at the 95% confidence level. There was no significant difference noticed for the Pittsburgh Green II method; therefore, this method proved to be more accurate than the BCG method for quantifying HSA in the standard human serum sample. The tendency for the BCG method to give higher HSA concentrations in sera was further corroborated when another commercial human serum sample was used; the BCG method and the Pittsburgh Green II method showed 57.4 ± 2.0 and 49.6 ± 2.0 g/L, respectively.

When HSA (125 μg; equivalent of 2.5 μL of human serum with 50 g/L HSA) was added to a pale yellow-green Pittsburgh Green II solution (1 mM, 2.0 mL), the solution turned red under ambient light (Figure 4a). Titration of human serum to a Pittsburgh Green II solution indicates a red shift in the absorption spectra (λ_{max}: 501 to 514 nm; Figure 4b) and emission spectra (λ_{max}: 522 to 534 nm; Figure 4c). Addition of HSA showed the same trend. As we stated above, we hypothesized that HSA-bound Pittsburgh Green II was protonated. To compare the HSA-bound dye and protonated dye, the solution of the dye was treated with acid; upon acidification, Pittsburgh Green II showed weaker absorption first and then a red shift (λ_{max}: 502 to 538 nm; Figure 4d). The acid-mediated red shift is highly unusual for xanthenone-based compounds, as DCF (Figure 4e) and **1** (Pittsburgh Green)[25] exhibited blue shifts in absorption spectra.

To further gain insights into the color change, we examined the solvatochromism of Pittsburgh Green II; a red shift was observed from MeOH (λ_{max} 512 nm) to DMSO (λ_{max} 532 nm) as a solvent (Figure 4f). Taken together, the addition of HSA to Pittsburgh Green II in water appears to protonate and/or dehydrate the dye (analogous to MeOH → DMSO), causing the red shift. The dehydration may account for fluorescence quenching, as fluorescein and DCF in the gas phase are essentially nonfluorescent.[30,31]

What is responsible for the unusual red shifts caused by HSA and acid? To address this question, we first discuss the protonation states of Pittsburgh Green II (**4**) (Figure 5). Upon acidification of a solution of **4**, the **4 + H** form is generated. Further acidification generates the cationic species **4 + 2H**$^+$. This cationic species would not be observed with DCF or **1** because an oxygen atom on the upper phenyl ring will react

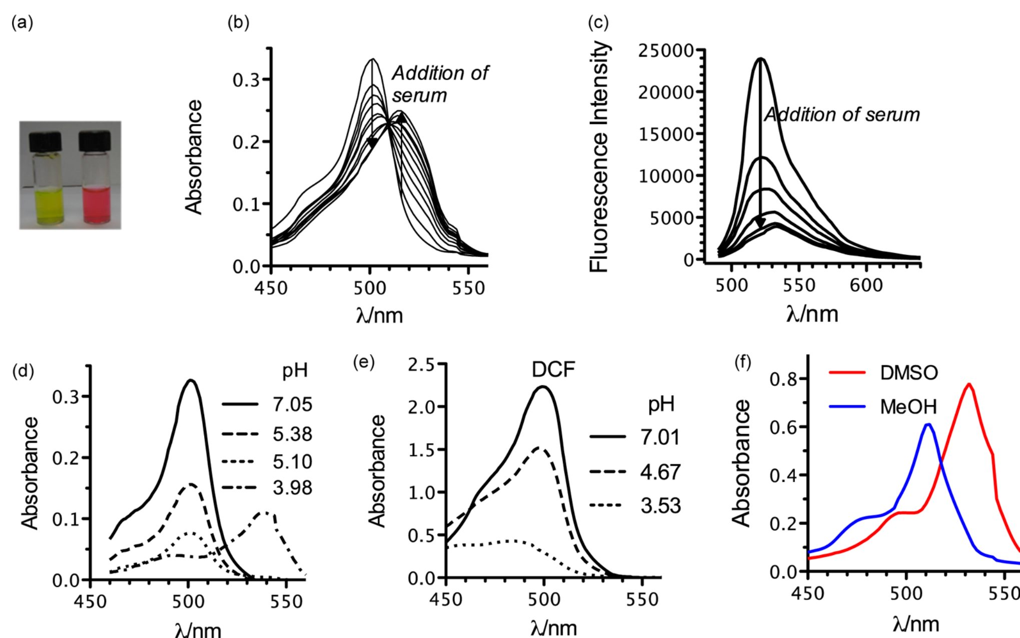

Figure 4. (a) Photographs of solutions of 1 mM Pittsburgh Green II in HEPES pH 7.0 buffer (2 mL) with HSA (125 μg; right) and without HSA (left). (b) UV−Vis spectra of Pittsburgh Green II with titration of human serum. [Pittsburgh Green II] = 80 μM (1.0 mL), human serum: 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 μL, 0.8% DMSO in pH 7 HEPES buffer, 25 °C. (c) Emission spectra of Pittsburgh Green II with titration of human serum. [Pittsburgh Green II] = 80 μM (1.0 mL), human serum: 0, 10, 20, 30, 40, and 50 μL, 0.8% DMSO in pH 7 HEPES buffer, 25 °C. Excitation: 470 nm. (d) UV−vis absorption spectrum of Pittsburgh Green II at various pHs. [Pittsburgh Green II] = 80 μM. (e) UV−Vis absorption spectrum of DCF at various pHs. [DCF] = 80 μM. (f) Solvatochromism of Pittsburgh Green II in organic solvents. The data are normalized for 10 μM Pittsburgh Green II.

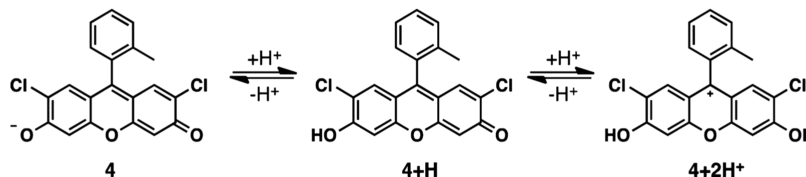

Figure 5. Three forms of Pittsburgh Green II (4).

with the carbocation to form a spirocyclic structure. Pittsburgh Green II does not contain a nucleophilic atom on the upper phenyl ring and thus can exist as the cationic species **4 + 2H**$^+$ under acidic conditions. The HSA-bound Pittsburgh Green II may possess the character of **4 + 2H**$^+$ as well. The presence of an isosbestic point shown in Figure 4b indicates that the binding of Pittsburgh Green II with HSA is reversible, supporting both hypotheses.[32]

In summary, we solved the long-standing problem associated with the BCG method by developing a new fluorometric method for HSA. The new method proved accurate and reproducible with the international standard serum sample, and the time-insensitivity provides a high-throughput means to measure HSA. This new method is >30 times more sensitive than the BCG method; the superior sensitivity may be exploited in the diagnosis of individuals with analbuminemia. An unusual red shift of a xanthenone derivative was discovered and caused by HSA and acid.

ASSOCIATED CONTENT

Supporting Information

Detailed protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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