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# Site-specific recognition of fluorescein by human serum albumin: A steady-state and time-resolved spectroscopic study

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ABSTRACT

The increased interest in using fluorescein as a fluorescent probe in biology and medicine is associated with its distinct absorption and fluorescence signals that are transparent to biological samples. Herein, we characterize the binding mechanism of fluorescein inside human serum albumin which is used as a carrier and protector for fluorescein in medical applications. Binding of fluorescein in human serum albumin causes partial fluorescence quenching of the sole tryptophan residue in the protein (W214). The estimated W214-fluorescein distance (2.42 nm) and the calculated quenching rate constant ($k_q = 5.13 \times 10^{12}$ $M^{-1}$ $s^{-1}$), both indicate binding of fluorescein in subdomain IIA. A site-competitive experiment shows that fluorescein is located in the warfarin binding pocket. The estimated binding constant ($K = 10{,}000$ $M^{-1}$) points to a moderate binding strength of the fluorescein–human serum albumin complex that should not affect the fluorescein release to the target when used as a probe.



## 1. Introduction

Fluorescein (FL) is widely used as an extrinsic label in proteins due to its distinct long absorption maximum near 490 with a high molar extinction coefficient, and emission wavelength from 480 to 600 nm with a high quantum yield [1,2]. These properties are significant in biochemical and biological applications which require a probe that possesses a fluorescence signal well removed from the intrinsic fluorescence of biological samples. One common use of FL is for labelling of antibodies. A wide variety of FL-labelled immunoglobulins are commercially available which are frequently used as proteins in fluorescence microscopy and in immunoassays [3–5]. The spectral properties of FL in the visible region make this molecule a suitable probe for such study and eliminate the need for quartz optics. FL is also used as a biological pH sensor to determine intracellular pH values. The molecule displays a complex pH-dependent equilibrium as shown in Scheme 1 [1,2]. The lactone form is usually found in organic solvents and is absent in aqueous solutions above pH 5.0. Only the monoanionic and the dianionic forms are fluorescent. The absorption spectrum shifts to longer wavelengths as the pH increases. These spectral changes allow wavelength-ratiometric pH measurements with two excitation wavelengths near 460 nm (monoanion) and 490 nm (dianion). The intensity ratio ($Int_{490}/Int_{460}$) increases with increasing pH.

Self-quenching of FL fluorescence has been reported even at low concentrations [6]. This is attributed to the very small Stokes shift which results in resonance energy transfer between nearby FL molecules (homo-transfer). As a result, the fluorescence intensity of a labelled protein decreases with the extent of labelling [7].

FL has been used as a fluorescent tracer in medical applications due to its high quantum yield in physiological conditions. It is used as a probe to measure the permeability of the human blood-ocular barriers [8,9]. However a rapid metabolic mechanism to a weakly fluorescent conjugate (FL-monoglucuronide) was reported [8,9]. In order to minimize the metabolic effect on FL, the molecule was used as a tracer after binding to human serum albumin (HSA) [10,11]. But when FL is administered systemically to study its rate of penetration through the blood-ocular barrier, the degree of its binding to albumin may affect the observed results. In this regard, a detailed characterization of the binding between FL and HSA is beneficial. In the present work, we characterize the binding properties of the HSA–FL complex using steady-state and time-resolved fluorescence measurements.

The HSA protein is one of the major carrier proteins in the body and constitutes approximately half of the protein found in human blood [12]. This protein of 585 residues is composed of a single polypeptide chain, with three α-helical domains I–III, each containing two subdomains A and B (Fig. 1) [13]. The protein is stabilized by 17 disulfide bridges. The crystal structure analyses indicate

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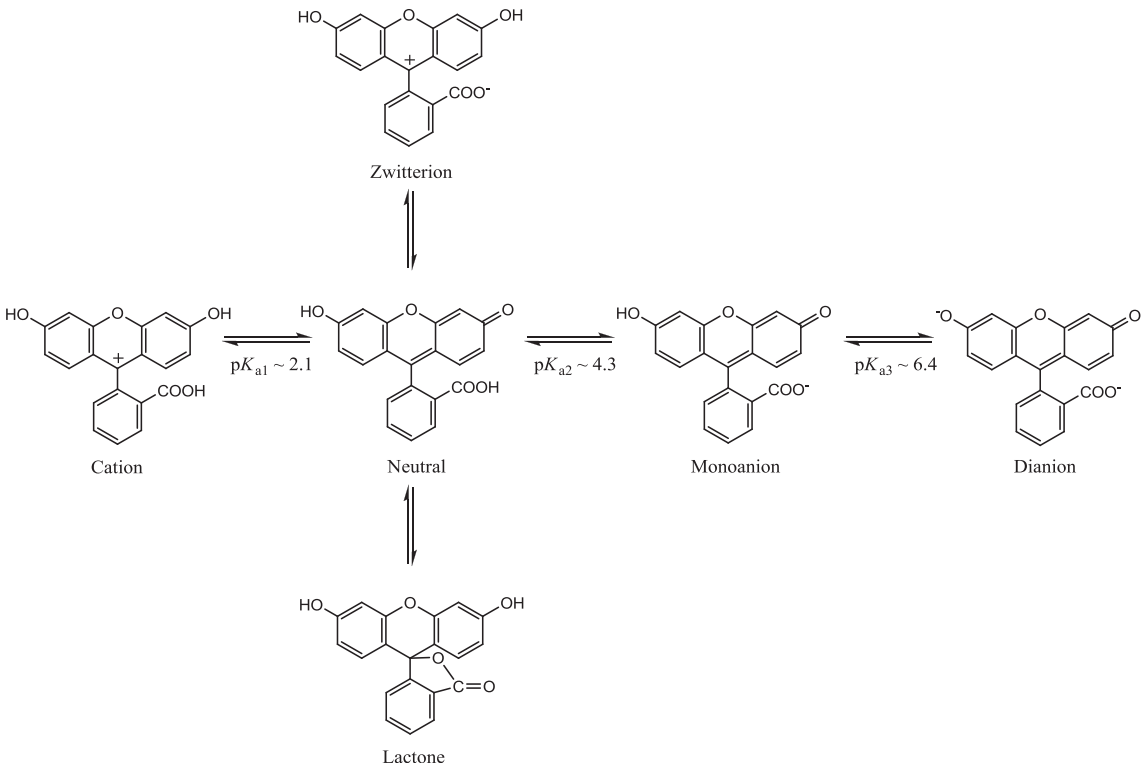


**Scheme 1.** Structures of different prototropic forms of fluorescein.

that HSA contains two primary drug-binding sites [13]. Site I (or the warfarin site) is located in subdomain IIA and prefers to bind large heterocyclic and negatively charged compounds, whereas Site II (or the indole-benzodiazepine site) is located in subdomain IIIA and is the preferred site for small aromatic carboxylic acids [14,15]. The sole tryptophan residue in the protein is located in Site I (W214) [14]. Herein, we show that FL specifically binds in Site I with a similar binding structure as that of warfarin. Among other parameters, we estimate the distance between FL and W214, and the binding constant from the fluorescence quenching of W214 by FL.

## 2. Experimental

Fluorescein and warfarin (98%) were purchased from Sigma–Aldrich and were used as received. HSA (essentially fatty acid free) was purchased from Sigma. The buffer used was 50 mM sodium phosphate (Aldrich), pH 7.2. Concentration of HSA in the buffer was prepared using its listed molecular weight of 66.5 kDa, and the final concentration was checked by comparing the measured absorbance with the published value (optical absorbance at 279 nm = 0.531 (1 g/L)) [16]. Deionized water (Millipore) was used in the preparations. The concentration of all materials was kept at 0.02 mM, unless otherwise mentioned.

Absorption spectra were obtained with an Agilent 8453 Diode Array UV–vis spectrophotometer. Fluorescence spectra were recorded on a Shimadzu RF-5301 PC spectrofluorophotometer. The fluorescence spectra were corrected for the difference in optical density at the excitation wavelength and inner filter effect [5,17].

Lifetime measurements were performed using a TimeMaster fluorescence lifetime spectrometer obtained from Photon Technology International. Excitation was at 295 and 435 nm using light-emitting diodes. In order to separate the fluorescence contribution of HSA from that of FL, a 340 nm CWL band-pass filter with 26 nm bandwidth (Edmund) was used. For the FL fluorescence, a cut-off filter (455 nm, Photon Technology International) was used. The system response time as measured from the scattered light was estimated to be approximately 1.5 ns. The measured transients were fitted to multi-exponential functions convoluted with the instrument response function (IRF). The fit was judged by the value of the reduced chi-squared ($\chi^2$). The experimental time resolution (after deconvolution) was approximately 100 ps, using stroboscopic detection [18].

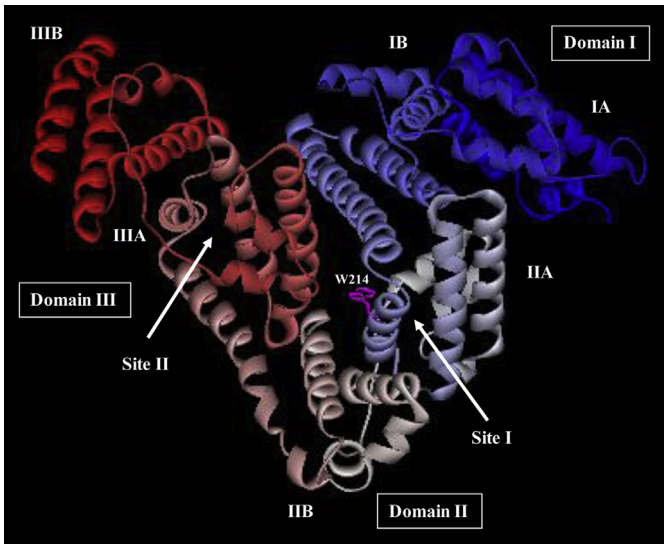


**Fig. 1.** The crystal structure of HSA and the locations of domain-binding sites. The locations of hydrophobic binding sites (Site I and Site II) are shown. The position of tryptophan residue (W214) in subdomain IIA is shown. The structure was obtained from the Protein Data Bank (ID code 1ha2).