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A red emissive donor-acceptor fluorophore as protein sensor: Synthesis, characterization and binding study

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ABSTRACT

The rational design of environmentally sensitive small molecule fluorophores with superior photophysical properties is critical for fluorimetry based biosensing. Herein, we have developed a new donor-acceptor fluorophore for quantitative detection of Human Serum Albumin (HSA) in aqueous samples. The fluorophore was easily prepared by Knoevenagel condensation, and showed excellent photophysical properties and positive solvatochromism. The design of the fluorophore was based on a nitrogen donor—π-conjugation—nitrile acceptors (D—π—A) to preserve efficient intramolecular charge transfer and long-wavelength emission. The fluorophore showed remarkable "turn-on" fluorescence in presence of HSA, which led to quantitative determination of the protein in aqueous buffer samples. Structure and electronic properties of the fluorophore played important roles on the superior HSA sensing ability. The findings indicate that minor changes in design strategy can be advantageous while developing long-wavelength (far red or near infrared) emitting fluorophores for biosensing and bioimaging.

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Introduction

Fluorimetric detection of biological molecules is of great importance in the field of biology and medicine [1–3]. Among many analytical techniques available fluorescence method stands out due to its high sensitivity and selectivity. Fluorometer is easy to operate, quick, and relatively inexpensive [3]. But, a suitable fluorophore is always required for quantitative detection of a target molecule in samples. Several promising new fluorophores have been described in literature, although selectivity and sensitivity could be improved [4–6].

In this context, environment sensitive fluorophores are very useful as "turn-on" fluorescent probes [7–12]. Such fluorophores can be inserted within a protein's cavity by simple solution mixing. However, optimum stereoelectronic and steric properties will play major roles in this process. Upon complexation, restricted movements and specific interactions within the protein's microenvironment alter photophysical properties of the fluorophore, which result in distinguishable and measurable spectral signals [3]. Moreover, a greater sensitivity is obtained when studies are performed in the near-infrared region (NIR, 650–900 nm), as scattering of light and interference from other biological molecules in samples remain minimal [13–19].

Herein, we have designed and reported a new far red emissive donor-acceptor (D—π—A) fluorophore (Scheme 1). An efficient intramolecular charge transfer (ICT) between the highly positive mesomeric donor (-NMe_2) and the powerful electron acceptor (tricyanofuran, TCF) has enabled quantitative detection of a protein, Human serum Albumin (HSA), in the red region (~650 nm) of the spectrum [20]. The fluorescence signal was intensified upon inclusion of the fluorophore within the protein's pocket; a tighter supramolecular complexation impeded non-radiative decay by restricting rotational degrees of freedom and hence increased the quantum yield of the fluorophore.

HSA is the most abundant protein in blood plasma. It is an important biomarker that can indicate a wide range of health conditions such as renal disease, coronary artery disease, stroke, and liver or kidney failure, especially for those with diabetes [21–25]. Therefore, a simple and cost-effective fluorimetric method for quantitative detection of HSA in samples is highly desirable.

Here, a small molecule D—π—A fluorophore with a polar $-CH_2CH_2OH$ group has enabled milligram level detection of HSA by fluorescence spectroscopy in aqueous buffer samples. The fluorophore binds selectively with HSA driven by hydrophobic and van der Waals type interactions. Lack of any aggregate formation in aqueous solution has resulted spontaneous supramolecular complex formation with HSA. In physiological buffer condition, detection limit for HSA was 30 nano-molar, which may be useful in point-of-care medical diagnosis in near future.

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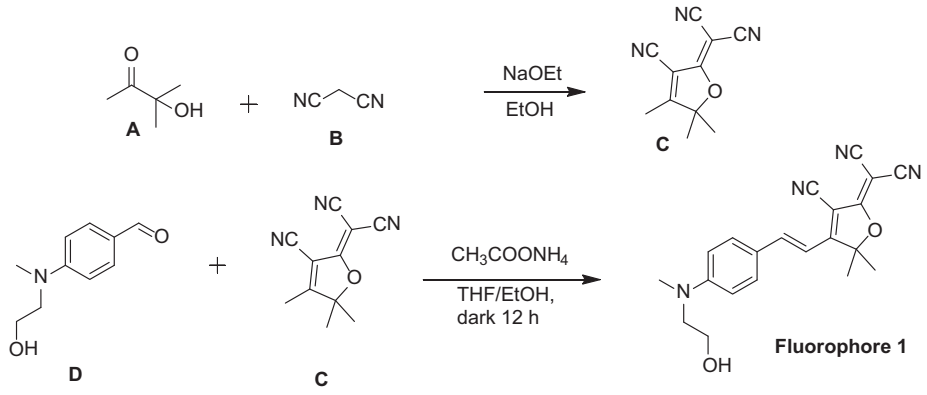

Scheme 1. Synthesis route for fluorophore **1**.

Results and discussion

Fluorophore **1** was synthesized following a literature report as shown in Scheme 1 [26]. In the first step 3-hydroxy-3-methylbutan-2-one (**A**) was coupled to malononitrile (**B**) to yield 2-(3-cyano-4,5,5-trimethylfuran-2(5H)-ylidene)malononitrile (**C**) as off-white powder (yield 48.4%). Knoevenagel condensation between **C** and 4-((2-hydroxyethyl)(methyl)amino)benzaldehyde (**D**) provided target fluorophore **1** in 44.5% yield. Detailed synthesis procedure and characterization results (FTIR, ^{1}H and ^{13}C NMR, ESI data, and elemental analysis) are provided in the Supporting information. The selectivity of Knoevenagel condensation to all-trans isomer was very high within the limits of NMR detection (Figs. S1 & S2). Presence of vinylic protons was confirmed from NMR coupling constant ($J \approx 16$ Hz) and the purity of the compound was confirmed by mass and elemental analysis.

Fluorophore **1** was highly soluble in polar aprotic solvents such as, acetonitrile, dichloromethane, dimethylsulfoxide (DMSO), dimethylformamide (DMF) as well as in polar protic solvents such as, ethanol, methanol, and isopropanol. It had minimal solubility in water at physiological pH.

Solution of fluorophore **1** exhibited different colors in different solvents (Fig. S3) [27,28]. For instance, pink in toluene, ethyl acetate, and acetone; blue in acetonitrile, DMF, and DMSO; purple in ethanol, methanol, and water. Fig. 1a shows absorption spectra of **1** ([**1**] = 1.0×10^{-5} M) in ten different solvents including water at pH 7.4 (λ_{max} = 587 nm; ε = 40,000 M^{-1} cm^{-1}). Absorption maxima (λ_{max}) in nonpolar solvents such as toluene and ethyl acetate were at 562 nm and 565 nm, respectively. With increased polarity of the solvents λ_{max} bathochromically shifted, indicating positive solvatochromism (Table S1). The longest absorption maxima recorded was in DMSO (λ_{max} = 595 nm). All these bands most likely arise from n–π^* electronic transition with an intramolecular charge transfer (ICT) from donor –NMe_2 to acceptor TCF [27].

Emission of **1** was recorded concurrently in the same set of solvents. Fig. 1b shows broad emissions of **1** in ten different solvents, ranging from yellow (in toluene, λ_{max} = 614 nm) to NIR-I region (in DMSO, λ_{max} = 665 nm) of the spectrum. Bathochromic shift was observed as polarity of the solvents increased, confirming positive solvatochromism of **1** (Table S2) [8,27]. Excitation spectra were recorded simultaneously to confirm that no fluorescent impurities were present in solution. Nice overlap with absorption spectra ruled out the possibility of presence of any secondary fluorescent species.

To be suitable for application in biological samples, fluorophore **1** must ideally remain as monomer in water. Therefore, aggregate formation of **1** was examined in water by recording absorption spectra with varying concentrations (up to 2.0×10^{-5} M) and then calculating molar extinction coefficients (ε) at those concentrations. Identical ε values indicate that **1** primarily remains as monomer in aqueous solution. Therefore, the emission spectrum of **1** in water ([**1**] = 1.0×10^{-5} M; pH = 7.4; λ^{em}_{max} = 647 nm) was recorded and adopted as working concentrations for all the experiments, unless otherwise stated. Moreover, absorption and emission spectra remained unchanged in the pH range of 4.0–10.0, indicating good pH tolerance of fluorophore **1** for potential biological applications (Fig. S4).

Fluorescence of donor-π-acceptor fluorophores is highly environment sensitive [8,12]. Due to intramolecular charge transfer a

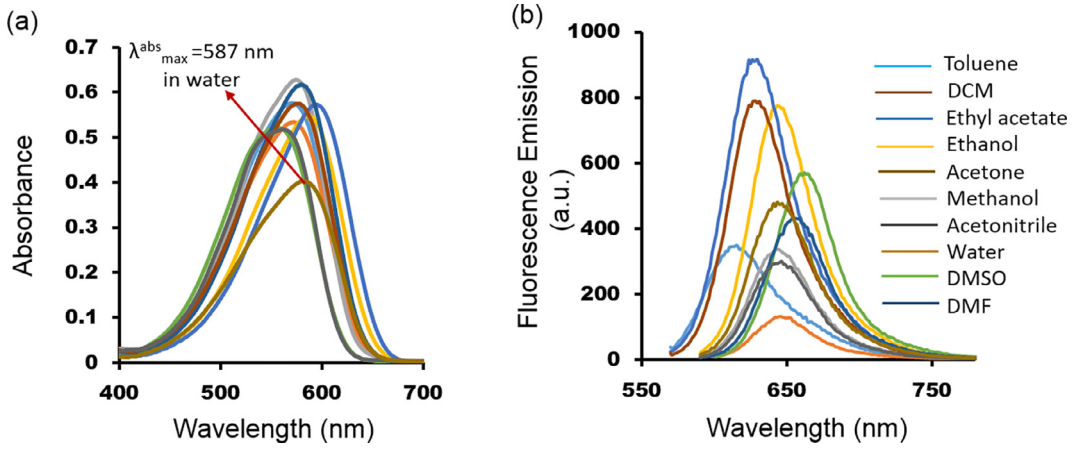

Fig. 1. (a) Absorption spectra of fluorophore **1** in various solvents. [**1**] = 1.0×10^{-5} M. Aqueous solution contains 1% DMSO. (b) Emission spectra of **1** in various solvents. [**1**] = 1.0×10^{-5} M. Aqueous solution contains 1% DMSO.

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