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Functional Molecular Lumino-Materials to Probe Serum Albumins: Solid Phase Selective Staining Through Noncovalent Fluorescent Labeling

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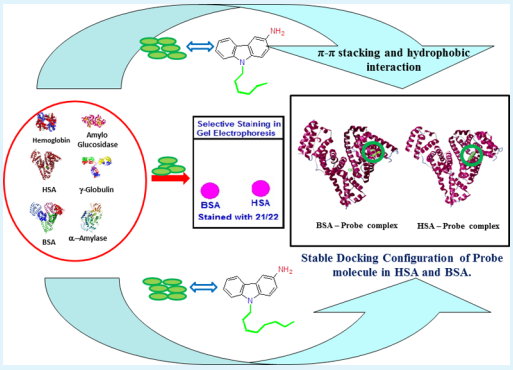
Supporting Information

ABSTRACT: Selective staining of human serum albumin protein in gel electrophoresis over wide range of other protein(s) is extremely important because it contains more than 60% volume of serum fluid in human body. Given the nonexistence of suitable dye materials for selective staining of serum albumins in gel electrophoresis, we report a new class of easy synthesizable and low molecular weight staining agents based on 3-amino-*N*-alkyl-carbazole scaffold for selective staining of serum albumins in solid phase. A detailed structure−efficiency relationship (SER) study enabled us to develop two such potent functional molecular probes which stain both human and bovine serum albumin selectively in gel electrophoresis in the presence of other proteins and enzymes. The present gel staining process was found to be very simple and less time-consuming as compared to the conventional coomassie blue staining which in turn makes these probes a new class of serum albumin-specific staining materials in proteome research. Moreover, these molecular lumino-materials can detect serum albumins at subnanomolar level in the presence of broad spectrum of other proteins/enzymes in aqueous buffer (99.9% water, pH = 7.3) keeping the protein secondary structure intact. Our experimental and the docking simulation results show that these probes bind preferentially at 'binding site I' of both the serum proteins.



KEYWORDS: *selective staining, serum albumins, gel electrophoresis, structure−efficiency relationship, molecular probes*

1. INTRODUCTION

Because of their paramount importance in regulating different biological events including various states of diseases, there is a pressing need for improved protein assays in proteome research.[1−4] Since its discovery, polyacrylamide gel electrophoresis has been proven to be one of the most efficient bioanalytical methods for successful detection and separation of proteins.[5−7] Among the various agents that are employed for staining different proteins in gel electrophoresis, small molecule-based organic fluorophores are widely used due to their high sensitivity, selectivity, and easy preparation. Therefore, efforts are being directed to develop efficient molecular probes.[8,9] However, and to the best of our knowledge, there is no report on molecular probes for selective staining of serum albumins in gel electrophoresis in the presence of other different proteins and enzymes. Hence, in addition to their potential in detecting serum albumins selectively in solution phase,[10−12] it is highly desirable to develop efficient molecular probes that can selectively stain serum albumins in solid phase. Specially, detection and staining of human serum albumin (HSA) in biological media is extremely crucial as the plethora of literature reports witness the great importance of this serum protein. HSA being the most abundant protein in human serum plays important role in shuttling of broad range of endogenous and exogenous ligands and transporting multiple fatty acids throughout entire human body.[13−16] In addition, it helps in scavenging free radicals,[17] maintaining acid base balance of plasma,[18] acts as a molecular chaperone preventing protein aggregation and amyloid formation,[19] and influences the vascular permeability to a certain extent.

In recent times, few small-molecule based fluorescent probes have been developed for the selective detection of serum albumins in solution phase.[10,18,20−24] Many of these probes require the presence of higher percentage of organic solvent to make them functioning in mixed aqueous solution[20,21,23,25] and their potential as selective protein/enzyme staining agent in gel electrophoresis has not yet been reported.

In this paper, we report a new class of easy synthesizable carbazole based functional organic lumino-materials with low molecular weight that selectively detect and stain serum

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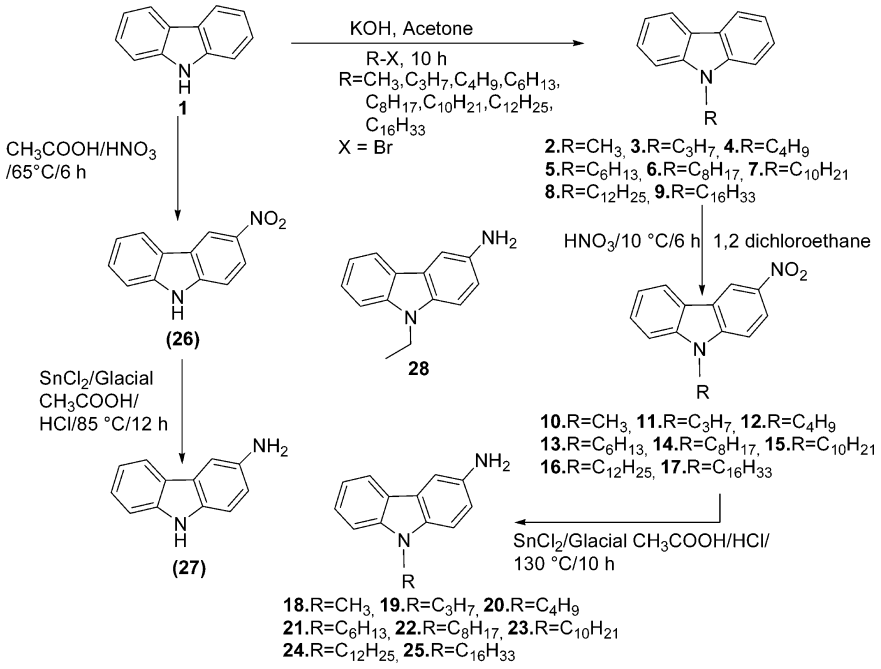
Scheme 1. Synthesis Strategy for the Preparation of Carbazole-Based Probes



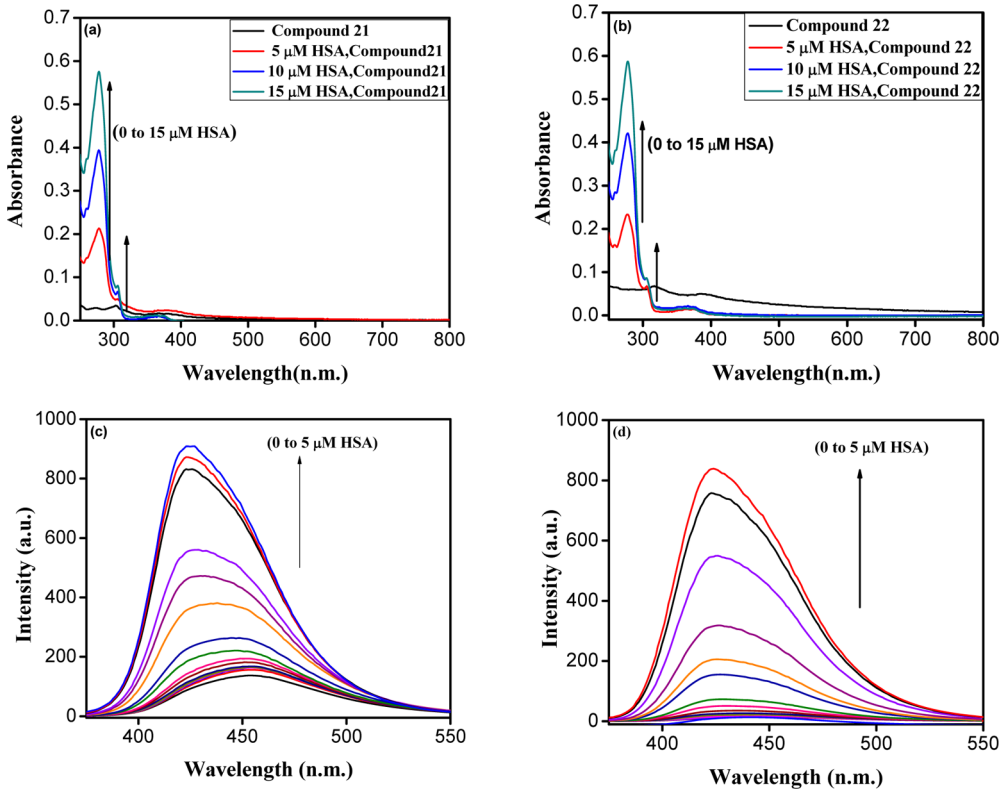


Figure 1. UV−vis and fluorescence emission spectra of **21**/**22** in the presence and absence of human serum albumin (HSA). Absorption spectra of 5 μM (a) **21** (b) **22** as a function of HSA (0 to 15 μm), and fluorescence emission spectra of 5 μM (c) **21**, (d) **22** as a function of HSA (0 to 5 μm) (arrows indicate increase in fluorescence intensity with gradual addition of HSA).

albumins at nanomolar level in the presence of several other proteins and enzymes (Supporting Information (SI): Figures S1 and S2; Table S1). The strong affinity of these newly developed molecular probes toward serum albumins enables postincubation staining in gel electrophoresis. The present probes were found to be much simpler and offer less time-consuming staining process than the other commonly used dyes for the post incubation staining.[26] The affinity of **21**/**22** toward HSA is comparable to CBBR250 and they generate sharper stained band than CBBR when protein bands having same concentration of HSA are stained with **21**/**22**. The probes work efficiently in 99.9% water solution. Because of the

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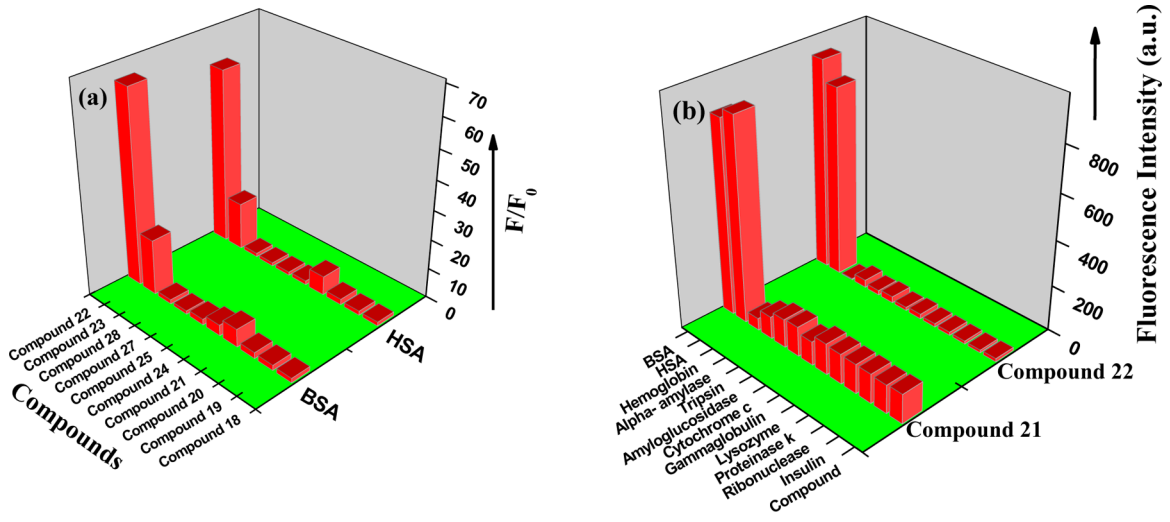

Figure 2. (a) Fluorescence emission spectra of different probes in the presence of BSA/HSA (5 μM). (b) Fluorescence emission spectra of **21**/**22** in the presence of various proteins and enzymes (5 μM).

structural similarity of bovine serum albumin (BSA) with HSA, these dyes also detect and stain BSA very efficiently. This suggests that the structure and chemical functionality play an important role in the detection and staining (SI Figure S3) of BSA/HSA.

2. EXPERIMENTAL SECTION

2.1. General Experimental. All chemicals required for the syntheses were purchased from commercial sources and used as received. Proteins and enzymes were purchased from Sigma-Aldrich. Freshly distilled solvents (*n*-hexane and ethyl acetate) were used for column chromatography and Merck silica gel (60−120 mesh) was used as stationary phase. Merck silica gel 60-F-254 plates were used for TLC. Compound **28** was purchased from Sigma-Aldrich. Instruments details have been provided in Supporting Information.

2.2. Synthesis. The present molecular probes were synthesized in three steps: alkylation of carbazole followed by nitration and finally reduction of nitro-derivatives to get the amine based end products. Supporting Information contains full experimental details and spectral characterization data.

3. RESULTS AND DISCUSSION

The design for developing carbazole based molecular probes was based on the combination of an amine functionality as electrostatic interaction unit and a hydrophobic tail to ensure interaction with the hydrophobic region of albumins. Initially two molecular probes, **20** and **28**, were used to investigate their affinity toward serum albumins. Whereas **28** was purchased from commercial source, **20** was synthesized following a three-step synthesis process (Scheme 1). Interestingly, the fluorescence signal observed due to probe-HSA/BSA interaction was found to be much stronger in **20** than **28** indicating the importance of hydrophobic tail to result in effective interaction with HSA and thus rendering strong optical signal (SI Figure S4). These results encouraged us to develop a new chemical library of such carbazole-based molecular probes and to study the structure−efficiency relationship (SER) to obtain the most potent probe for detecting serum albumins.[20] The synthetic procedures that were followed while developing this library are outlined in Scheme 1. In brief, carbazole **1** was alkylated first to get *N*-alkyl carbazoles **2**−**9** followed by their nitration to obtain the corresponding nitro-derivatives **10**−**17**.[27] These nitro-derivatives were then further reduced using $SnCl_2$ as reducing agent to get the final desired amines **18**−**25**.[28] To investigate the importance of alkyl chains, 3-aminocarbazole **27** was also synthesized (Scheme 1).

While evaluating their potentials, compound **21** and **22** were found to be the most potent ones among all these newly developed probes. The UV−vis spectra of both the compounds **21**/**22** (5 μM) in a mixture of water/DMSO (99.9:0.1) were almost similar possessing absorption bands at 295 (ε = 6322.58), 302 (ε = 7554.25) and 347 (ε = 3900.29) nm for **21**, and 295 (ε = 12128.32), 315 (ε = 13935.48), and 347 (ε = 10903.22) nm for **22** (Figure 1a and b, SI Figure S5A and B). Whereas a prominent increase in the absorption intensity at 280 nm was observed without any spectral shift upon the addition of serum albumins to a solution of **21**, the addition of serum albumins to a solution of **22** resulted in a 13 nm blue shift to the 315 nm absorption band with a gradual increase in intensity (Figure 1a and b, SI Figure S5A and B). Moreover, the gradual addition of HSA to a solution of **21**/**22** also resulted in slight increase in absorption intensity at 302 nm (Figure 1a and b.

While investigating the effect of fluorescence emission in water/DMSO (99.9:0.1) mixture, both the compounds (5 μM) were observed to have weak fluorescence property (Figure 1c, SI Figure S5C and D). Moreover, probe **21** was found to be much stronger fluorescent material than **22** (λ_{ex} = 302 nm, λ_{em} = 453 nm) (Figure 1d, SI Figure S5C and D). The addition of either BSA or HSA to these probe solutions resulted in fluorescence enhancement with around 30 nm bathochromic shift. While a 6-fold increase in fluorescence emission was observed for **21**, almost 66-fold increment in fluorescence intensity was observed for **22** upon the addition of BSA or HSA (Figure 1c and d, SI Figures S5C and D, S6A−D). Though the fluorescence enhancement was instantaneous in case of **21**, almost 45−50 min incubation was necessary to obtain the highest fluorescence intensity in case of **22**. This is possibly due to the presence of longer alkyl chain which brings in steric factor while interacting with BSA/HSA and therefore 22 needs longer time to reach to the binding site. We also performed similar experiments with other compounds of this newly developed chemical library. However, neither the shorter nor the longer alkyl chain compounds (**18**, **19**, **23**, **24**, **25**, **28**) showed such high extent of fluorescence emission except **20**

(Figure 2a, SI Figure S6A–D). Further, probe **27** (without any alkyl chain but contains amine functionality) and carbazole **1** itself (without any alkyl chain or amine functionality) also did not show such strong fluorescence enhancement (SI Figure S7). The association constants for BSA-**21**/**22** complexes were calculated to be 2.5×10^{-11} and 2.8×10^{-11} M^{-2}, respectively (SI Table S1, SI Figure S8).[29,30] Similarly, the association constants for HSA-**21**/**22** complexes are 3.05×10^{-11} and 2.15×10^{-11} M^{-2} respectively (SI Table S1). These association constants were calculated using 1:2 (protein/probe) stoichiometry, which was confirmed from fluorescence titration data (SI Figure S8).[29−31] To check the specificity of the above two compounds (**21**/**22**), a large number of different proteins and enzymes were used (insulin, hemoglobin, tripsin, proteinase K, α-amylase, amyloglucosidase, γ-globulin, lysozyme, ribonuclease A, and cytochrome C). Interestingly, no such fluorescence enhancement was observed when **21**/**22** intereacted with any of these proteins/enzymes. These data exclusively indicated the specificity of **21**/**22** toward serum albumins (Figure 2b, SI Figure S9). The negligible affinity of **22** toward other proteins/enzymes is possibly due to the absence of suitable hydrophobic cleft.[20] No change in fluorescence emission of **21**/**22** was observed in the presence of free tryptophan amino acid (SI Figure S10). These experimental data revealed the importance of protein microenvironment to bring in such strong change in fluorescence emission of **21**/**22**. The nanosecond lifetime data for **21**/**22** in the absence of BSA/HSA showed biexponential decay with a lifetime of major contribution of 95.32% and 86.65% respectively (SI Figure S11) (nonradiative decay constants K_{nr}, in the presence and absence of serum albumins, are given in SI Table S2). These results revealed that the nonradiative decay rate of both the compounds decreased significantly in the microenvironment of the proteins (SI Table S2), which in turn led to the increment in fluorescence intensity of these probes in the presence of serum albumins. This was supported further by the anisotropy data. The increase in anisotropy of **21**/**22** was 3−4 folds in the presence of serum albumins due to their tight binding in the microenvironment of proteins (SI Table S2).

The previous discussion discloses the importance of the alkyl chain for these newly developed functional molecular materials in order to be BSA/HSA specific. We were also curious to investigate the importance of the amine functionality in the detection of BSA/HSA. To this endeavor, the ligand binding of amine free *N*-alkylcarbazole derivatives **2**−**9** to BSA/HSA was investigated. The characteristic fluorescence emission envelop of all the probes **2**−**9** fall within the same region as of the tryptophan emission of BSA/HSA, however with an additional red-shifted pick (SI Figure S12A). Though a many fold increase in fluorescence intensity of **4**/**5** (respective amine derivatives are **21**/**22**) was observed in the presence of BSA/HSA, these enhancements included free BSA/HSA fluorescence (SI Figure S12A). Hence, a subtracted fluorescence was calculated to know the actual enhancement in fluorescence intensity for the amine free derivatives. This indicates that the amine free molecules can also be used to detect serum albumins, but this might lead to the erroneous conclusion while analyzing fluorescence enhancement. Therefore, **21**/**22** were chosen as model compounds for all sorts of further studies as their fluorescence maxima were far apart from the tryptophan fluorescence. Interestingly, the nitro-derivatives **10**−**17** did not result any such fluorescence enhancement in the presence of BSA/HSA (SI Figure S12B). Therefore, the presence of amine and suitable alkyl units was essential for these probes to be protein specific and to generate emission peak which is different from the emission of BSA/HSA.

Next, we investigated the effect of pH and salt concentration on the labeling efficiency of these probes in solution phase. It was observed that **21**/**22** are efficient enough in rendering turn-on signaling in a wide pH range 6.5−11, which actually covers the physiological range (pH 7−9) (SI Figure S13). Similarly, the effect of salt concentration was also investigated using different strength of buffer (1×, 2.5×, 5×, 7.5×, and 10×). The salt concentration that is required for preparing 10X buffer was found to result in maximum fluorescence enhancement (SI Figure S14).

The sensing efficiencies of these newly developed probes **21**/**22** were not affected by the fatty acids (SI Figure S15). The experiments were performed using isovaleric acid as model fatty acid. As it is well-known that in addition to other biological entities blood plasma also contains various fatty acids, these results demonstrate that **21**/**22** can be used for sensing serum albumins in real samples without any interference of fatty acids.

After optimizing the experimental conditions to obtain maximum fluorescence enhancement, the calibration graphs were plotted using the data obtained by titrating BSA/HSA with **21**/**22** (SI Figures S1 and S2), and the results are documented in SI Tables S3 and S4.[32−36] The high sensitivity and efficiency of these newly developed probes encouraged us to investigate their potential in quantifying serum albumin present in blood serum. The detail of this quantification process has been provided in SI (Figure S16, Tables S5−S7). We observed that the quantification results were very similar to the results obtained using standard methods that are followed in the pathology center indicating the strong potential of these probes for practical application. A wide linear range 0.025−0.6 μM was observed in the calibration plots (SI Figure S16).[37−39]

The photostability of **21**/**22** was initially investigated in the laboratory, well-lighted with common fluorescent tubes/lamps and windows, at room temperature. Both the probes were found to be stable more than 3 days with almost equal sensing efficiency (SI Figures S17 and S18). This stability was comparable with stability in the absence of light (SI Figures S17 and S18). We also investigated the photostability of **21**/**22** in the presence of strong and direct sunlight. Though some extent of degradation was observed when interacted with the sunlight directly, the stability and sensing were observed up to 1 h (SI Figures S19 and S20). All these results clearly indicate that **21**/**22** have good photostability.

It was necessary at this point to get insights into the probable binding sites of the proteins for these probes, which might help to understand the binding mechanism as well. The site selectivity of these probes was studied using two very well-known site specific dyes *viz* phenylbutazone (for site I) and dansyl proline (for site II). As these two known dyes bind strongly to the two different sites of the BSA/HSA, a ligand displacement strategy was very helpful to understand the binding sites.[40] While 18% and 22% displacements were observed upon the addition of phenylbutazone to **22**-BSA and **22**-HSA complexes respectively, 91% and 89% displacements were observed in the presence of dansyl proline (SI Figure S21).[40] Similarly, the presence of phenylbutazone caused 30% and 22% displacements to the **21**-BSA and **21**-HSA complexes, respectively, whereas the presence of dansyl proline resulted in 81% and 76% displacements respectively (SI Figure S22).[40] All these experimental results indicated that both the probes

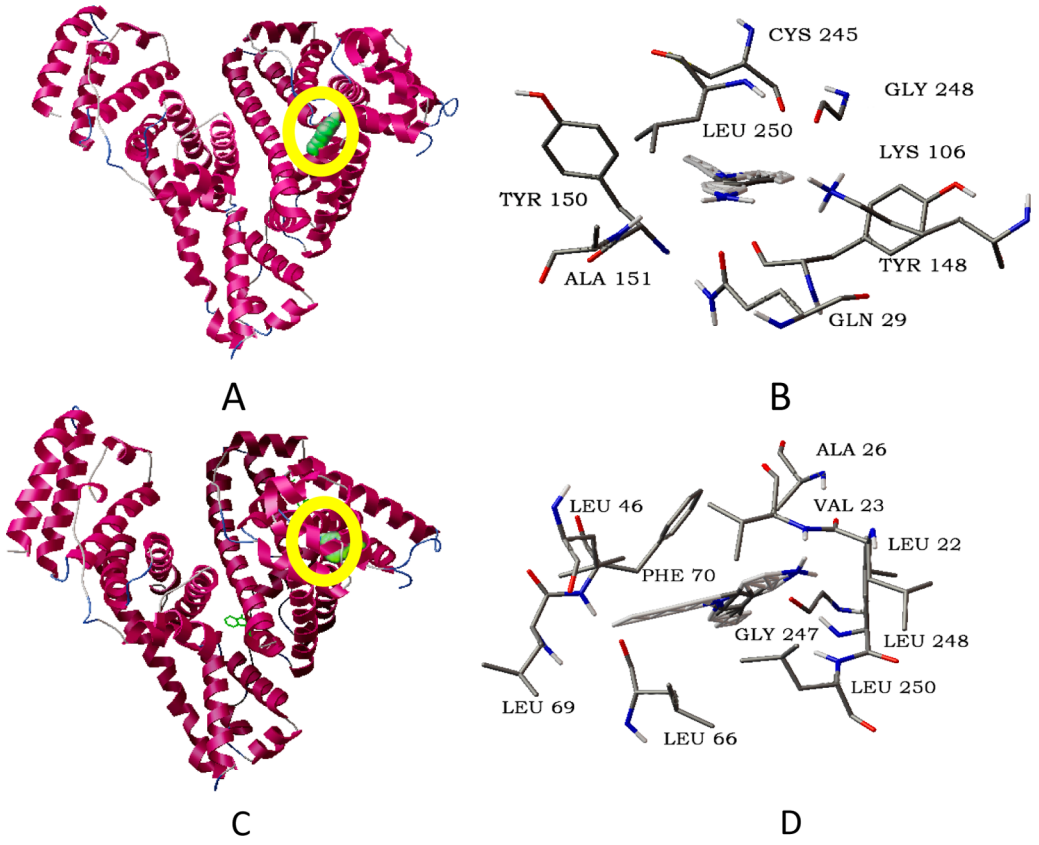

Figure 3. Docking simultion results on the probe binding and structural conformation of **21** binding inside the protein pocket. (A and C) Most stable docking configurations of **21** in HSA and BSA, respectively; (B and D) amino acid residues of the proteins that surround the probe **21**.

preferentially bind at site I, with a little chance to bind at site II. This binding is possibly due to $\pi-\pi$ stacking and strong hydrophobic interaction. The above conclusion was further supported by the molecular docking simulation.[41,42] Both the compounds were docked with both HSA and BSA proteins using standard AutoDock 4.2 tools. The AutoDock 4.2 based docking technique includes a search over the entire surface of the protein for binding sites. The lowest binding energy conformer has been searched out of 10 different conformations for each docking simulation and the resultant minimum energy one has been exploited for further analysis.[42−44]

The docking results indicate a strong binding of both the compounds **21** and **22** at site I specifically (IIA domain of either BSA or HSA) (Figure 3, SI Figures S23 and S24). A little probability for site II was also observed for both the compounds, however the earlier found to be major conformation which was also supported by the ligand displacement reaction. The close view of the docking results (<1 Å) shows that the binding is mainly due to hydrophobic interaction and hydrogen bonding (Figure 3, SI Table S8).

Finally, for real biological application it was extremely important to check the structural stability of both the proteins in the presence of **21**/**22**. Circular dichroism (CD) is the most powerful technique to understand the secondary structural conformation of a protein while attached to a ligand. Our data suggested that the native α-helix structure of proteins did not get affected in the presence of these molecular probes (SI Figure S25). Therefore, this light-up fluorescence signaling process was completely nondetrimental to the native structure of these proteins.

As these probes showed strong affinity toward serum albumins resulting in turn-on fluorescence signaling, we next investigated the potential of these probes as selective staining agents for BSA/HSA in gel electrophoresis. For this investigation native PAGE technique and comassie blue were used as standard control.[11] We observed that both the probes **21**/**22** successfully stained BSA/HSA in gel electrophoresis proving their potential as new cheap selective staining dyes for serum albumins (Figures 4 and 5). The staining and developing process was much easier and less time-consuming than comassie blue. Whereas coomassie blue staining involves several post separation steps for successful stain development,[26] stains using probe **21**/**22** were developed simply by treating the gel after separation with the probes solutions followed by watching under UV transilluminator (post incubation technique) (detailed procedure is given in Supporting Information). Next, we investigated the selectivity of the dye **22** toward staining BSA/HSA in the presence of other proteins/enzymes. Probe **22** was found to stain selectively serum albumins in the presence of other proteins/enzymes (Figure 4, lanes f, g, h, and i). To the best of our knowledge, this is the first dye for selective staining of serum albumins in solid phase. To check the sensitivity of this dye, staining of different concentrations of serum albumins was investigated and we observed that the dye **22** was sensitive enough to stain 100 ng of BSA per band (SI Figures S26 and S27). Even the efficiency of this dye in staining albumins present in the real serum was tested and the probe **22** successfully stained the albumins of a freshly collected serum sample (SI Figure S28). Similar to the solution phase labeling, serum albumins were successfully stained in the solid phase with these probes in wide pH range 7−11 (SI Figure S29).

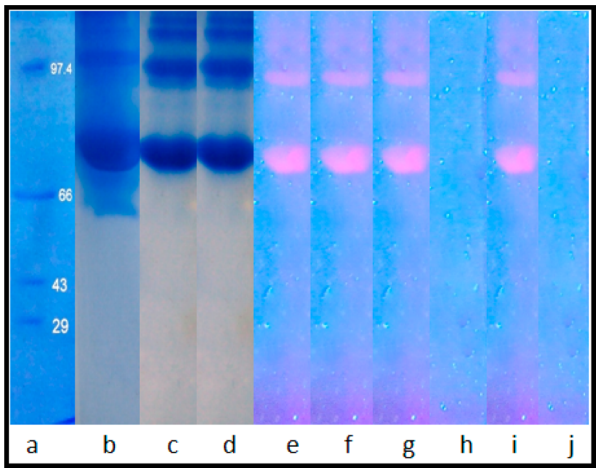

Figure 4. Selective staining: Native discontinuous PAGE of BSA, HSA, hemoglobin, glucosidase, γ- globulin, and α-amylase; In lanes b, c, and d the gel is stained with CBBR 250 after separation and in lanes e–j the gel was treated with **22** in 10× PBS after separation. Lane a: protein marker containing molecular weights of 97.4, 66, 43, and 29 kDa and the gel is stained with CBBR 250 after separation. Lane b: mixture of proteins + BSA (66.5 kDa). Lane c: BSA. Lane d: HSA. Lane e: BSA. Lane f: HSA. Lane g: mixture with BSA. Lane h: mixture without BSA. Lane i: mixture with HSA. Lane j: mixture without HSA.

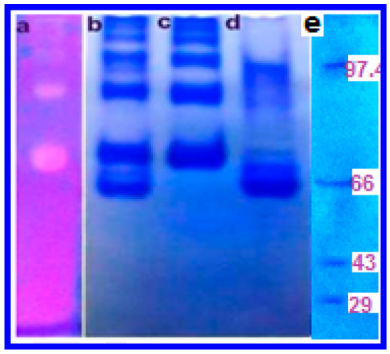

Figure 5. Selective staining. Lane a: mixture of egg white and BSA treated with **22** in 10× PBS buffer. Lane b: mixture of egg white and BSA. Lane c: BSA. Lane d: egg white. Lane e: protein marker containing molecular weights of 97.4, 66, 43, and 29 kDa. Lanes b–e stained with CBBR 250 after separation.

4. CONCLUSIONS

To conclude, careful structure–efficiency/turn-on fluorescence relationship studies have resulted in the development, for the first time, of novel small-molecule based dye materials that can stain serum albumins selectively in gel electrophoresis. Staining of very low concentration of proteins proves their high efficiency/sensitivity. In the same time, these probes were found to be efficient enough in detecting BSA/HSA in solution at nanomolar range through a turn-on fluorescence signaling in aqueous solution with ~70 folds increase in fluorescence intensity and 425% enhancement in fluorescence quantum yield. Dye displacement strategy and molecular docking experiments revealed that these probes preferentially bind to site I of BSA/HSA. These probes were successfully used for the quantification of HSA present in blood serum. We strongly believe that these albumin specific molecular probes will be find broad applications in proteome research.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

General information, experimental procedures, additional spectroscopy and gel staining results, scanned copies of $^{1}H/^{13}C$ NMR of all intermediates and final compounds are available. 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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