Application of Photoinduced Electron Transfer with Copper Nanoclusters toward Finding Characteristics of Protein Pockets

Soumyadip Bhunia, Sumit Kumar, and Pradipta Purkayastha
†Department of Chemical Sciences and ‡Center for Advanced Functional Materials (CAFM), Indian Institute of Chemical Sciences (IISER) Kolkata, Mohanpur 741246, West Bengal, India

ABSTRACT: Proteins possess various domains and subdomain pockets with varying hydrophobicity/hydrophilicity. The local polarities of these domains play a major role in oxidation–reduction-based biological processes. Herein, we have synthesized ultrasmall fluorescent copper nanoclusters (Cu NCs) that are directed to bind to the different domain-specific pockets of the model protein bovine serum albumins (BSA). Potential electron acceptors, methyl viologen (MV) derivatives, were chosen such that they specifically reach the various domains following their hydrophobicity/hydrophilicity. Here, we have used MV\(^{2+}\), HMV\(^{+}\), and DHMV\(^{3+}\), possessing hydrophilic, intermediate, and hydrophobic specificities. Being electron acceptors, these derivatives draw electrons from the Cu NCs through photoinduced electron transfer (PET). The rate of PET varies at the different domains of BSA based on the local environment which has been analyzed. Here, PET is confirmed by steady state as well as time-resolved fluorescence spectroscopy. This study would provide a measurable way to identify the location of the different domains of a protein which is scalable by changing the superficial conditions without unfolding the protein.

INTRODUCTION

The hierarchical structure of proteins is constructed by the steric constraints generated on the arrangements of the amino acids. The chiral nature and the hydrogen bonding properties of the backbone create the secondary structure of proteins that generally contains repetition of α-helices and β-sheets known as “motifs.” Assembly of protein motifs into larger subunits of structures, called “domains”, is often defined as a unit of conserved sequence. There can be structural and sequential similarity between proteins, such as human and bovine serum albumins (HSA and BSA). Hence, classification and comparison of a new protein structure can be done through identification and partitioning of multiple domains in a protein structure. In the two examples of proteins cited above, BSA is one of the most studied serum albumin because of its structural similarity to HSA and high water solubility. BSA consists of 583 amino acid residues comprised of three homologous helical domains [I(1−195), II(196−383) and III(384−583)] each having two subdomains. Seventeen disulfide bridges divide the homologous domains into nine loops to provide rigidity to the structure of BSA. α-helices predominate the BSA structure with no β-sheet. Site II of BSA is similar to HSA although Leu-237 residue occupies site I of BSA which is hollow for HSA. Thus the Leu-237 residue in site I of BSA prevents the insertion of hydrophobic molecules. The serum albumins bind exceptionally well with many endogenous and exogenous compounds, support distribution of ligands, and protect them from being metabolized. In addition to the various ways of serum albumin binding, their conformational dynamics toward diverse stimuli are studied as they control efficient delivery of drugs and provide knowledge on molecular level protein functions.

BSA, a typical serum albumin, binds to plenty of guests and provides information on the protein structure under various external conditions. Cooperative binding of different dyes with BSA and HSA provides important information on site-selective binding. Choice of suitable functional groups for the interacting guest helps to detect active sites of the proteins through noncovalent bonding. Electrostatic interaction of BSA and charged species can be investigated using ionic fluorescent probes that emit upon aggregation. It has also been shown that hydrogen bonding plays a vital role in binding amphiphiles to BSA.

From the above cited examples, it becomes clear that hydrophobic interaction preludes binding of different species to BSA at site I (cavity size of 2.53 Å) of subdomain II and binding at site II (cavity size 2.6 Å) involves hydrophobic, hydrogen bonding and electrostatic interactions. Molecular docking helps in identifying the potential binding pockets within the protein structure. A number of binding pockets of various sizes are present in the structure of BSA. The challenge to find the specific characteristic of the protein...
pockets of various hydroaffinities seems to have remained partly explored.\textsuperscript{21,22} Interaction of copper nanocluster (Cu NC) probes with serum albumins have been reported on the formation of protein corona over the Cu NCs\textsuperscript{23,24} and creating energy transfer antenna using HSA or L-cysteine (Cys)-protected Cu NCs.\textsuperscript{25,26} A previous study on interaction of Cu NCs with BSA showed minor loss in the secondary structure.\textsuperscript{27} However, there is hardly any report on recognition of protein pockets by electron-transfer mechanism. Hence, the model protein, BSA, has been chosen to establish this methodology using biocompatible luminescent Cu NC as probe.

To explore the different hydrophobic, hydrophilic, and mixed zones of BSA quantitatively, we have synthesized Cys-protected luminescent Cu NCs containing 9 Cu atoms and used photoinduced electron transfer (PET) between the Cu NCs and various methyl viologen (MV) derivatives which are well-known electron acceptors.\textsuperscript{28–30} Because there were several reports on protein-protected Au and Ag NCs and hence from the knowledge of biocompatibility, we have chosen Cu NCs for the present study.\textsuperscript{31}

For the present study, 9 Cu atom Cu NCs was drop-casted on a carbon-coated copper grid and dried in air. Electron-dispersive spectroscopy (EDS) was performed using a JEM-2100F field emission gun electron microscope equipped with EDS, diffraction pattern software, and high angle annular dark-field scanning TEM detector.

Zeta Potential Measurement. The \(\xi\)-potential measurements were performed in a nanoparticle analyzer SZ-100 from Horiba Scientific using He–Ne laser beam at 633 nm. An aqueous solution of Cu NCs was used for this experiment.

Fourier Transform Infrared Spectroscopy. IR spectra were recorded on a Bruker (model ALPHALPHA) FT-IR spectrometer. A KBr pellet was made by mixing requisite amount of sample with KBr.

Dynamic Light Scattering Measurement. A Malvern Zetasizer Nano equipped with a 4.0 mW HeNe laser operating at \(\lambda = 633\) nm was used for the dynamic light scattering (DLS) measurements at a scattering angle of 173°. Nonnegative least-square analysis was used to calculate the size distribution.

Steady-State Spectroscopy. The absorption and steady-state fluorescence spectral measurements were carried out using a Hitachi U-2900 spectrophotometer and a QM 40 spectrophluorimeter from PTI Inc., respectively. Comparing the wavelength-integrated intensity of the Cu NCs with the standard (quinine sulphate; \(Q_Y = 0.54\)) yielded the fluorescence quantum yield. The concentration-related artifacts were avoided by using solutions having absorbance (OD) less than 0.05. The excitation wavelength was 360 nm. Quantum yield was calculated using the following equation

\[
Q = Q_R \frac{\text{OD}_R I n^2}{\text{OD}_I \xi_R^2}
\]

where \(Q, \text{OD}, I, \) and \(n\) stand for quantum yield, absorbance, integrated luminescence intensity, and refractive index of the solvents, respectively. Subscript \(R\) stands for reference (standard dye).

Time-Resolved Measurement. A 375 nm diode laser excitation source (with a temporal resolution of 70 ps) was used in the time-resolved fluorescence experiments which were performed using a Horiba Jobin Yvon Fluorocube instrument. The experimental method adopted was time-correlated single photon counting (TCSPC). The fluorescence decay data were fitted with a proper exponential decay equation. The nonlinear least square iterative revolvement procedure was done using IBH DA6 (Version 2.2). The \(\chi^2\) values assessed the quality of the fit. PL decay traces have been observed to be multi-exponential in nature and could be successfully fitted with a biexponential or sometimes with a triexponential function

\[
I(t) = I(0) \sum_{i=1}^{n} A_i e^{-t/\tau_i}
\]
where $I(0)$ and $I(t)$ are the PL intensities at time 0 and $t$, respectively. Average lifetime value, $\langle \tau \rangle$, can be calculated from the fitted data using the following equation

$$\langle \tau \rangle = \frac{\sum A_i \tau_i^2}{\sum A_i \tau_i}$$

where $\tau_i$ is the excited-state lifetime of each component of PL decay and $A_i$ is the relative amplitude of that very component.

**Steady-State Fluorescence Anisotropy Measurement.**

Steady-state fluorescence anisotropy measurements were done in a QM-40 spectrofluorimeter from PTI using excitation and emission polarizers. The value of anisotropy ($r$) was calculated using the following equation

$$r = \frac{I_\parallel - G I_\perp}{I_\parallel + 2 G I_\perp}$$

where $G$ is the correction factor for the detector sensitivity to the polarization direction of the emission and $I_\parallel$ and $I_\perp$ are the fluorescence decays polarized parallel and perpendicular to the polarization of the excitation light, respectively.

**Rotational Anisotropy Measurement.**

Time-resolved rotational anisotropy data were collected via the same TCSPC setup using excitation and emission polarizers. Time-resolved fluorescence anisotropy, $r(t)$, was calculated using the following equation

$$r(t) = \frac{I_\parallel(t) - G I_\perp(t)}{I_\parallel(t) + 2 G I_\perp(t)}$$

where $G$ is the correction factor for the detector sensitivity to the polarization direction of the emission and $I_\parallel(t)$ and $I_\perp(t)$ are the fluorescence decays polarized parallel and perpendicular to the polarization of the excitation light, respectively.

Rotational correlation time ($\tau_{\text{rot}}$) was extracted by fitting the time-resolved anisotropy decay using a suitable exponential decay equation.

**Circular Dichroism Spectroscopy.**

The CD spectra were measured in a JASCO J-815 spectrometer using a quartz cuvette with 1 mm pathlength in the wavelength range 200–400 nm. A scan speed 100 nm/min was used to get the CD profiles.

**RESULTS AND DISCUSSION**

The Cys-protected Cu NCs were synthesized following a reported protocol with slight modification as shown in Scheme 1.26 The Cu$^{2+}$–Cys complex was formed on the addition of Cys to copper nitrate solution.35,36 At basic pH (pH $\approx$ 12), deprotonation of the thiol group in Cys takes place, facilitating the reduction of Cu(II) to Cu(I) and Cu(0) without any externally added reducing agent. At high pH, Cys can perform the dual role of reducing agent and surface protecting ligand. Detail of the synthetic method is provided in the Supporting Information. The synthesized Cu NCs were characterized microscopically as well as spectroscopically (Figure 1). From the TEM data, we found that the Cu NCs were spherical in shape with an average diameter of 1.94 ± 0.12 nm and well dispersed without any aggregation (Figure 1A,B). The mass of the Cu NCs was calculated using electrospray ionization mass spectrometry (ESI-MS) measurement (Figure 1C), where a peak at $m/z$ 857.87 appeared corresponding to [Cu$_9$L$_2$ + 2Na$^+$ + H$^+$]. Using the spherical jellium model, we determined the number of Cu atoms in the NCs from the spectral data. The model is applicable to small metal NCs and is mathematically represented as

$$\Delta E_{\text{emission}} = \frac{E_{\text{Fermi}}}{N^{2/3}}$$
where $\Delta E_{\text{emission}}$ is the energy of emission from the fluorescing NCs, $E_{\text{Fermi}}$ is the Fermi energy, and $N$ represents the number of metal atoms in NCs. Calculations (see Supporting Information, Section 3) show that the cyan-emitting Cu NCs contain nine Cu atoms in agreement with the ESI-MS measurement.

Fourier transform infrared (FTIR) spectroscopy provided the surface functionalities of the Cu NCs (Figure S1A). The thiol groups of Cys protect the metal atoms through covalent bond formation. The FTIR spectrum of pure Cys shows a small peak at 2580 cm$^{-1}$ because of the S–H stretching. This peak completely disappears on formation of the Cu NCs, confirming the formation of Cu–S covalent bonds. On the other hand, –NH$_2$ stretching produces a broad peak in the range 3000–3800 cm$^{-1}$. Additionally, broad peaks in the range of 1300–1660 cm$^{-1}$ indicate the presence of –COOH groups. These peaks remained unaltered in the spectrum for Cys–Cu NCs, indicating the existence of the –NH$_2$ and –COOH groups in NCs as well. Hence, the FTIR study confirmed that the Cu atoms in the Cu NCs were capped by the thiol moieties from Cys ligands. An apparent zeta potential value of $-58.6$ mV indicated negative surface charge of the Cys–Cu NCs (Figure S1B). This highly negative surface charge value is due to the presence of the terminal carboxylate ions of Cys. In alkaline medium, the Na$^+$ ions from NaOH pair up with the carboxylate groups through electrostatic interaction, providing

Figure 2. Optical characterization of the Cys–Cu NCs: (A) absorption, excitation, and emission spectrum (inset: zoomed image of the absorption spectrum in the range 300–450 nm to show the shoulder at 362 nm indicated by the black arrow); (B) excitation of the sample at different wavelengths showed that the Cu NCs are monodispersed in solution phase. The experiments were performed at 24 °C.

Figure 3. MV and its derivatives used in the present experiment.

Figure 4. Quenching of fluorescence from the Cu NCs by (A) DHMV$^{2+}$, (B) HMV$^+$, and (C) MV$^{2+}$. The samples were excited at 370 nm. The Stern–Volmer plots are assimilated in (D) for the three quenchers.
greater solubility to the Cys–Cu NCs in aqueous medium. DLS measurement showed the average hydrodynamic diameter of the Cys–Cu NCs as ∼7.5 nm in an aqueous medium ([Figure S1C]). The absorption spectrum was typically featureless as that for noble metal NCs besides a broad hump around ∼362 nm ([inset of Figure 2A]). The absence of any peak around 500–600 nm confirms the absence of surface plasmon resonance and hence Cu nanoparticles (Cu NPs) and formation of Cu NCs ([Figure 2A]). Additionally, the excitation spectrum of Cu NCs is consistent with an absorption spectrum. Upon exciting at 370 nm, Cu NCs give intense cyan color with emission maximum at 485 nm. The excitation wavelength-independent photoluminescence (PL) emission maxima ([Figure 2B]) indicate the discreteness and specificity, ensuring the monodispersity and molecule-like behavior of the Cu NCs. The fluorescence quantum yield of the synthesized Cu NCs is calculated using quinine sulphate (Φ = 0.54) in 0.1 M H2SO4 as standard. The obtained quantum yield is 0.078 for the Cys–Cu NCs.

The synthesized Cys–Cu NCs were used as reporter fluorophores for the various pockets of BSA and the signal of PET between the MV derivatives and the NCs was used to quantify the behavior of the NCs in the protein pockets. We chose three types of MV derivatives, viz., 1,1′-diheptyl-4,4′-bipyridinium dibromide (DHMV2+), 1-heptyl-4-(4-pyridyl)pyridinium bromide (HMV+), and the parent methyl viologen (MV2+), for this purpose ([Figure 3]). As mentioned earlier that the different subdomains of BSA have sites that bind specifically to either hydrophobic or hydrophilic guests and also to sites which have mixed characteristics, we chose DHMV2+, HMV+, and MV2+ for binding to the hydrophobic, mixed, and hydrophilic pockets, respectively. PET from metal NCs to MVs is well known, and plenty of works are reported in this area. The three derivatives exhibit difference in the extent of electron acceptance from a common donor because of the presence of the attached hydrophobic chains and hence variation in the electron deficiency at the acceptance center. The initial PET experiments with the Cu NCs and the MV derivatives were performed to estimate their respective PET capacity so that it can be compared with those obtained in the presence of BSA.

The absorption spectrum of Cu NC (0.1 mM) did not show much change ([Figure S2]) on addition of the MV derivatives. However, MV2+ and its derivatives quench the fluorescence from the Cu NCs to different degrees ([Figure 4A–C]) presumably because of PET from the Cu NCs to the MV derivatives. This quenching is supposed to be dynamic, which was also confirmed from the respective Stern–Volmer plot ([Figure 4D]). The extent of fluorescence quenching is determined following the Stern–Volmer equation

\[
\frac{F}{F_0} = 1 + K_{SV}[Q]
\]

where \(F_0\) and \(F\) are the fluorescence intensities without and with the quencher, \(K_{SV}\) is the Stern–Volmer constant, and \([Q]\) is the concentration of quencher. A straight line plot indicates one specific type of quenching (either static or dynamic). Considering the corresponding excited-state lifetime, the Stern–Volmer equation can also be expressed as

\[
\frac{\tau_0}{\tau} = 1 + K_{SV}[Q]
\]

and

\[
K_{SV} = k_q \tau_0
\]

where \(\tau_0\) and \(\tau\) are the excited-state lifetimes of the fluorophore in the absence and presence of a quencher and \(k_q\) is the rate constant for the quenching process. Time-resolved fluorescence decay shows that progressive change in the lifetime of the Cu NCs on adding the MV derivatives and linear fit to \(\tau_0/\tau\) against quencher concen-
Fluorescence by the MV Derivatives

The quantum yield of the donors can be determined by comparing the emission spectrum of the donor with the absorption spectrum of the acceptor. In this way, the overlap integral can be calculated. There is no overlap integral for the MV derivatives, as there is no overlap between the emission spectrum of the MV derivatives and the absorption spectrum of the Cu NCs.

The extent of PET can be monitored to obtain a quantitative idea on the impact of the protein pockets on the Cu NCs due to the specific environments. The absorption spectra obtained due to interaction between the Cu NCs and BSA did not show much change at higher wavelengths. Absorbance at 278 nm due to BSA increases on increasing the protein concentration (Figure S3). We excited the Cu NCs bound to BSA to avoid any data contamination due to energy transfer between the tryptophan residues of BSA and the Cu NCs. On addition of BSA to the Cu NCs, there is a slight increase in the Cu NC emission with a little blue shift, indicating protein–Cu NC interaction (Figure S4).

The steady-state anisotropy (r) of Cu NC increased on interacting with BSA (Figure S5) and the rotational freedom decreased as obtained from the time-resolved anisotropy decay measurements (Figure S6). The value of τrot increased from 0.12 to 0.28 ns with the increase in BSA concentration, confirming attachment of Cu NCs with BSA. From the Benesi–Hildebrand double reciprocal plots, we observed that the Cu NCs bind to BSA neither in 1:1 nor 1:2 ways as the fits deviate from linearity in both cases (Figure S7). The result is contrary to that reported by Sahu et al.27 However, the attachment of the Cu NCs does not change the secondary structure of BSA appreciably as can be seen from the CD spectra (Figure S8). Moreover, the results indicate that binding of the Cu NCs is not restricted only to the surface hydrophilic pockets of BSA but also extends to the inner moderate and less hydrophilic ones.

The emission peak of the Cu NCs shows about 10 nm blue shift on addition of BSA due to the change in polarity of the environment around the NCs (Figure 6). This indicates possible encapsulation of a portion of the Cu NCs to enter the hydrophobic zones of BSA. Fluorescence of the Cu NCs bound in the BSA pockets is quenched by the MV derivatives as they reach specifically to the destinations based on their hydrophobicity/hydrophilicity (Figure 6A–C). The Stern–Volmer plot shows straight line fits for each of the three cases, indicating one specific type of quenching (dynamic quenching as per the above discussion) (Figure 6D). Comparison of the quenching trends in BSA with those in bulk water shows lesser PET in the trapped state. Because the MV derivatives shall reach the destination BSA pockets specifically depending on the hydro-availability, the quenching parameters need to be compared for each of the quenchers.

Table 1. Decay Parameters for Cu NC in Water in the Presence of DHMV2+, HMV+, and MV2+14

| MV derivatives | concentration (mM) | τ1 (ns) | B1 (%) | τ2 (ns) | B2 (%) | ⟨τ⟩ (ns) | χ2 |
|----------------|--------------------|---------|--------|---------|--------|----------|-----|
| DHMV2+        | 0                  | 1.42    | 5      | 10      | 95     | 9.93     | 1.13|
|                | 2                  | 1.11    | 6      | 7.64    | 94     | 7.58     | 1.09|
|                | 4                  | 0.62    | 6      | 6.19    | 95     | 6.16     | 1.08|
|                | 6                  | 0.8     | 6      | 5.35    | 94     | 5.32     | 1.11|
| HMV+          | 0                  | 1.42    | 5      | 10      | 95     | 9.93     | 1.13|
|                | 2                  | 1.4     | 7      | 8.91    | 93     | 8.83     | 1.07|
|                | 4                  | 1.28    | 9      | 7.8     | 91     | 7.70     | 1.12|
|                | 6                  | 0.99    | 9      | 6.84    | 91     | 6.76     | 1.16|
| MV2+          | 0                  | 1.42    | 5      | 10      | 95     | 9.93     | 1.13|
|                | 2                  | 1.21    | 6      | 7.76    | 94     | 7.70     | 1.09|
|                | 4                  | 0.8     | 6      | 6.25    | 94     | 6.21     | 1.15|
|                | 6                  | 0.66    | 6      | 5.41    | 94     | 5.38     | 1.05|

“χ2 values show the goodness of the fits. ⟨τ⟩ represent the mean lifetimes. The samples were excited at 375 nm and the 488 nm emission was monitored.

The Cu NCs were found to readily interact with BSA. Unlike D. K. Sahu and K. Sahu, who synthesized 14-atom Cu NCs and studied their interaction with BSA inferring 1:1 binding,27 and Das et al., who reported adsorption of HSA on glutathione-protected Cu NCS, we could identify the protein pockets. Moreover, 1:1 and 1:2 host–guest binding in BSA can be well-understood from Benesi–Hildebrand double reciprocal plots.31,42 On contrary to the reported protein–Cu NC studies, besides mere guest–host binding, we intended to look into the characteristics of the domain specific pockets in BSA. Hence, we synthesized the Cys-protected ultrasmall fluorescent Cu NCs intending their attachment to the various protein pockets so that, by sending environment specific electron withdrawing probes (the MV derivatives), the extents of PET can be monitored to obtain a quantitative idea on the specific environments.

The Cu NCs bind to BSA neither in 1:1 nor 1:2 ways as the fits deviate from linearity in both cases (Figure S7). The results indicate attachment of multiple Cu NCs to BSA, conforming with the proposed binding to multiple protein pockets. This result is contrary to that reported by Sahu et al.27 However, the attachment of the Cu NCs does not change the secondary structure of BSA appreciably as can be seen from the CD spectra (Figure S8). Moreover, the results indicate that binding of the Cu NCs is not restricted only to the surface hydrophilic pockets of BSA but also extends to the inner moderate and less hydrophilic ones.
The time-resolved studies show small change in the average lifetime of the Cu NCs on interaction with BSA (Figure 7A–C and Table 3). A third ultrafast component ($\tau_1$) evolved on addition of BSA to the Cu NCs because of binding of the Cu
The phenomenon provides us information on the electron acceptors used in the form of MV derivatives and the rate of increase or decrease in already containing Cu NCs. On reaching the Cu NCs, the MV intermediate, and least hydrophobic pockets of BSA that is assume that these ions move specifically to the most, intermediate, and least hydrophobic pockets of BSA that is already containing Cu NCs. On reaching the Cu NCs, the MV derivatives undergo PET and the rate of increase or decrease in the phenomenon provides us information on the effect of the environment on the Cu NCs and hence the BSA pockets. These information are important, as they would let us know how the specific structure(s) of biological (protein) redox systems modulate rates and specificities of physiological redox processes. Possibility of protein designing to minimize these rapid rates to stop disastrous biological "short circuits" might evolve by knowing the changes.42

Comparing the data in Tables 2 and 4 and from the $K_{sv}$ and $k_0$ values, we could calculate the change in the extent of PET from the Cu NCs in free and bound states. Considering the solvation characteristics of DHMV$^{2+}$, HMV$^+$, and MV$^{2+}$, we assume that these ions move specifically to the most, intermediate, and least hydrophobic pockets of BSA that is already containing Cu NCs. On reaching the Cu NCs, the MV derivatives undergo PET and the rate of increase or decrease in the phenomenon provides us information on the effect of the environment on the Cu NCs and hence the BSA pockets. These information are important, as they would let us know how the specific structure(s) of biological (protein) redox systems modulate rates and specificities of physiological redox processes. Possibility of protein designing to minimize these rapid rates to stop disastrous biological "short circuits" might evolve by knowing the changes.42

Cu NCs in the hydrophobic pockets of BSA lead to 32% decrease in PET compared with that in bulk water which accompanies 26% lowering in the quenching rate. The changes are quite similar in hydrophilic pockets where PET from Cu NC to MV$^{2+}$ decreases by 35% and the quenching rate lowers by 29%. The reduction in PET is much less (20%) in the pockets with intermediate hydrophobicity (or hydrophilicity), and the quenching rate lowers by 13%. The values indicate that the rate of oxidation–reduction in the protein pockets of different hydrophobicity (or hydrophilicity) depend much on the environment.

**CONCLUSION**

The results of interaction of minute fluorescent Cys-protected Cu NCs with the site-specific MV derivatives in the protein pockets having various polarities show that the quantitative measures of the extent of local oxidation–reduction would allow one to modulate the protein structure scaling to the right need. The Cu NCs used in this study are not specific to surface binding and diffuse to all the protein pockets. Site-specific electron acceptors were used in the form of MV derivatives, and the phenomenon of PET could be used to quantify the characteristics of the pockets. In the vast array of studies on drug–protein and nanoparticle–protein interactions, the local specification of the protein pockets were left unknown. Our studies could provide a measurable way to understand the characteristic of the different domain specific pockets of a protein which is scalable by changing the superficial conditions without unfolding the protein.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b03213.

Detail characterization of the synthesized Cu NCs (PDF)

**AUTHOR INFORMATION**

**Corresponding Author**

*E-mail: ppurkayastha@iiserkol.ac.in (P.P.).

**ORCID**

Soumyadip Bhunia: 0000-0002-5276-7807

Pradipta Purkayastha: 0000-0001-8825-1207

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

The financial support from the Science and Engineering Research Board of the Department of Science and Technology, Government of India through project number EMR/2015/000950 is gratefully acknowledged. S.B. and S.K. acknowledge financial support from the Science and Engineering Research Board of the Department of Science and Technology, Government of India through project number EMR/2015/000950. Y.M. acknowledges the support from the Ministry of Science, Technology, and Innovation Malaysia through FRGS grant 203/PFIZIK/6101658.
the Council of Scientific and Industrial Research and Indian Institute of Science Education and Research Kolkata for their fellowships.

REFERENCES

(1) Levitt, M.; Chothia, C. Structural Patterns in Globular Proteins. Nature 1976, 176, 552–558.
(2) Marchler-Bauer, A.; Anderson, J. B.; Derbyshire, M. K.; DeWeese-Scott, C.; Gonzalez, N. R.; Gwadz, M.; Hao, L.; He, S.; Hurwitz, D. I.; Jackson, J. D.; Ke, Z.; Krylov, D.; Lancycky, C. J.; Liebert, C. A.; Liu, C.; Lu, F.; Lu, S.; Marchler, G. H.; Mullokandov, M.; Song, J. S.; Thamki, N.; Yamashita, R. A.; Yin, J. J.; Zhang, D.; Bryant, S. H. CDD: a conserved domain database for interactive domain family analysis. Nucleic Acids Res. 2007, 35, D237–D240.
(3) He, X. M.; Carter, D. C. Atomic Structure and Chemistry of Human Serum Albumin. Nature 1992, 358, 209–215.
(4) Dufour, C.; Dangles, O. Flavonoid-Serum Albumin Complexation: Determination of Binding Constants and Binding Sites by Fluorescence Spectroscopy. Biochim. Biophys. Acta Gen. Subj. 2005, 1721, 164–173.
(5) Yang, F.; Zhang, Y.; Liang, H. Interactive Association of Drugs Binding to Human Serum Albumin. Int. J. Mol. Sci. 2014, 15, 3580–3595.
(6) Varshney, A.; Sen, P.; Ahmad, E.; Rehan, M.; Subbarao, N.; Khan, R. H. Ligand Binding Strategies of Human Serum Albumin: How Can the Cargo be Utilized? Chirality 2010, 22, 77–87.
(7) Krug-Hansen, U. Molecular and Practical Aspects of the Enzymatic Properties of Human Serum Albumin and of Albumin-Ligand Complexes. Biochim. Biophys. Acta Gen. Subj. 2013, 1830, 5535–5544.
(8) de Wolf, F. A.; Brett, G. M. Ligand-Binding Proteins: Their Potential for Application in Systems for Controlled Delivery and Uptake of Ligands. PharmacoI. Rev. 2000, 52, 207–236.
(9) Yang, F.; Bian, C.; Zhu, L.; Zhao, G.; Huang, Z.; Huang, M. Effect of Human Serum Albumin on Drug Metabolism: Structural Evidence of Esterase Activity of Human Serum Albumin. J. Struct. Biol. 2007, 157, 348–355.
(10) Mallick, A.; Halder, B.; Chattopadhyay, N. Spectroscopic Investigation on the Interaction of ICT Probe 3-acyetyl-4-oxo-6,7-dihydro-12H Indol-2-[3,4-a] quinolizine with Serum Albumins. J. Phys. Chem. B 2005, 109, 14683–14690.
(11) Anand, U.; Mukherjee, S. Binding, Unfolding and Refolding Dynamics of Serum Albumins. Biochim. Biophys. Acta Gen. Subj. 2013, 1830, 5394–5404.
(12) Cai, H.-H.; Zhong, X.; Yang, P.-H.; Wei, W.; Chen, J.; Cai, J. Probing Site-Selective Binding of Rhodamine B to Bovine Serum Albumin. Colloids Surf., A 2010, 372, 35–40.
(13) Jisha, V. S.; Arun, K. T.; Harirahan, M.; Ramaiah, D. Site-Selective Interactions: Squaraine Dye–Serum Albumin Complexes with Enhanced Fluorescence and Triplet Yields. J. Phys. Chem. B 2010, 114, 5912–5919.
(14) Abe, Y.; Fukui, S.; Koshiji, Y.; Kobayashi, M.; Shoji, T.; Sugata, S.; Nishizawa, H.; Suzuki, H.; Iwata, K. Enantioselective Binding Sites on Bovine Serum Albumin to Dansyl Amino Acids. Biochim. Biophys. Acta Protein Struct. Mol. Enzymol. 1999, 1433, 188–197.
(15) Yang, M.; Wu, Y.; Li, J.; Zhou, H.; Wang, X. Binding of Curcumin with Bovine Serum Albumin in the Presence of t-Carrageenan and Implications on the Stability and Antioxidant Activity of Curcumin. J. Agric. Food Chem. 2013, 61, 7150–7155.
(16) Suzuki, Y.; Yokoyama, K. Design and Synthesis of Intramolecular Charge Transfer-Based Fluorescent Reagents for the Highly-Sensitive Detection of Proteins. J. Am. Chem. Soc. 2005, 127, 17799–17802.
(17) Tong, J.; Hu, T.; Qin, A.; Sun, J. Z.; Tang, B. Z. Depolarizing the Binding Behaviours of BSA Using Ionic AIE-Active Fluorescent Probes. Faraday Discuss. 2017, 196, 285–303.
(18) Ghosh, S.; Dey, J. Binding of Fatty Acid Amide Aphiophiles to Bovine Serum Albumin: Role of Amide Hydrogen Bonding. J. Phys. Chem. B 2015, 119, 7804–7815.
(19) Jisha, V. S.; Arun, K. T.; Harirahan, M.; Ramaiah, D. Site-Selective Binding and Dual Mode Recognition of Serum Albumin by a Squaraine Dye. J. Am. Chem. Soc. 2006, 128, 6024–6025.
(20) Ma, L.-J.; Li, Y.; Li, L.; Sun, J.; Tian, C.; Wu, Y. A Protein-Supported Fluorescent Reagent for the Highly-Sensitive and Selective Detection of Mercury Ions in Aqueous Solution and Live Cells. Chem. Commun. 2008, 6345–6347.
(21) Coleman, R. G.; Sharp, K. A. Protein Pockets: Inventory, Shape, and Comparison. J. Chem. Inf. Model. 2010, 50, 589–603.
(22) Stank, A.; Kokh, D. B.; Fuller, J. C.; Wade, R. C. Protein Binding Pocket Dynamics. Acc. Chem. Res. 2016, 49, 809–815.
(23) Hsieh, S.-R.; Reddy, P.; Chang, C.-J.; Kumar, A.; Wu, W.-C.; Lin, H.-Y. Exploring the Behavior of Bovine Serum Albumin in Response to Changes in the Chemical Composition of Responsive Polymers: Experimental and Simulation Studies. Polymers 2016, 8, 238.
(24) Das, N. K.; Chakraborty, S.; Mukherjee, M.; Mukherjee, S. Enhanced Luminescent Properties of Photo-Stable Copper Nanoclusters through Formation of “Protein-Corona”–Like Assemblies. ChemPhysChem 2018, 19, 2218–2223.
(25) Ghosh, S.; Das, N. K.; Anand, U.; Mukherjee, S. Photostable Copper Nanoclusters: Compatible Förster Resonance Energy-Transfer Assays and a Nanometer. J. Phys. Chem. Lett. 2015, 6, 1293–1298.
(26) Maity, S.; Bain, D.; Bhattacharyya, K.; Das, S.; Bera, R.; Jana, B.; Paramanik, B.; Datta, A.; Patra, A. Ultrafast Relaxation Dynamics of Luminescent Copper Nanoclusters (Cu7L3) and Efficient Electron Transfer to Functionalized Reduced Graphene Oxide. J. Phys. Chem. C 2017, 122, 13354–13362.
(27) Sahu, D. K.; Sahu, K. Characterizing Optical Properties, Composition of Stabilizer-Free Copper Nanoclusters and its Interaction with Bovine Serum Albumin. J. Photochem. Photobiol., A 2017, 347, 17–25.
(28) Chen, W.-T.; Hu, Y.-J.; Kamat, P. V. Realizing Visible Photoactivity of Metal Nanoparticles: Excited-State Behavior and Electron-Transfer Properties of Silver (Ag8) Clusters. J. Phys. Chem. Lett. 2012, 3, 2493–2499.
(29) Aly, S. M.; AbdulHalim, L. G.; Besong, T. M. D.; Soldan, G.; Bakr, O. M.; Mohammed, O. F. Ultrafast static and diffusion-controlled electron transfer at Ag29 nanocluster/molecular acceptor interfaces. Nanoscale 2016, 8, 5412–5416.
(30) Mondal, S.; Purkayastha, P. α-Cyclodextrin Functionalized Carbon Dots: Pronounced Photoinduced Electron Transfer by Aggregated Nanostructures. J. Phys. Chem. C 2016, 120, 14365–14371.
(31) Das, S.; Purkayastha, P. Gold Nanocluster Protection of Protein from UVC Radiation: A Model Study on Bovine Serum Albumin. ACS Omega 2017, 2, 2451–2458.
(32) Jana, J.; Acharya, P.; Negishi, Y.; Pal, T. Evolution of Silver-Mediated, Enhanced Fluorescence Au-Ag Nanoclusters under UV Activation: A Platform for Sensing. ACS Omega 2018, 3, 3463–3470.
(33) Nandi, I.; Chall, S.; Chowdhury, S.; Mitra, T.; Roy, S. S.; Chattopadhyay, K. Protein Fibril-Templated Biomimetic Synthesis of Highly Fluorescent Gold Nanoclusters and Their Applications in Cysteine Sensing. ACS Omega 2018, 3, 7703–7714.
(34) Abraham, A. N.; Sharma, T. K.; Bansal, V.; Shukla, R. Phytochemicals as Dynamic Surface Ligands To Control Nanoparticle-Protein Interactions. ACS Omega 2018, 3, 2220–2229.
(35) Luo, Z.; Nachammai, V.; Zhang, B.; Yan, N.; Leong, D. T.; Jiang, D.-e.; Xie, J. Toward Understanding the Growth Mechanism: Tracing All Stable Intermediate Species from Reduction of Au(I)-Thiolate Complexes to Evolution of Au25 Nanoclusters. J. Am. Chem. Soc. 2014, 136, 10577–10580.
(36) Chakraborty, I.; Erusapppan, J.; Govindarajan, A.; Sugi, K. S.; Udaiyabhaskaran, T.; Ghosh, A.; Pradeep, T. Emergence of
Metallicity in Silver Clusters in the 150 Atom Regime: A Study of Differently Sized Silver Clusters. *Nanoscale* 2014, 6, 8024–8031.

(37) Lin, Z.; Slee, T.; Mingos, D. M. P. A Structural Jellium Model of Cluster Electronic Structure. *Chem. Phys.* 1990, 142, 321–334.

(38) Seidl, M.; Brack, M. Liquid Drop Model for Charged Spherical Metal Clusters. *Ann. Phys.* 1996, 245, 275–310.

(39) Bhunia, S.; Kumar, S.; Purkayastha, P. Gold Nanocluster-Grafted Cyclodextrin Suprastructures: Formation of Nanospheres to Nanocubes with Intriguing Photophysics. *ACS Omega* 2018, 3, 1492–1497.

(40) Wei, W.; Lu, Y.; Chen, W.; Chen, S. One-Pot Synthesis, Photoluminescence, and Electrocatalytic Properties of Subnanometer-Sized Copper Clusters. *J. Am. Chem. Soc.* 2011, 133, 2060–2063.

(41) Kundu, P.; Chattopadhyay, N. Unraveling the Binding Interaction of a Bioactive Pyrazole-Based Probe with Serum Proteins: Relative Concentration Dependent 1:1 and 2:1 Probe-Protein Stoichiometries. *Biophys. Chem.* 2018, 240, 70–81.

(42) McLendon, G. Long-Distance Electron Transfer in Proteins and Model Systems. *Acc. Chem. Res.* 1998, 21, 160–167.