Optical Spectroscopic and Morphological Characterizations of Curcuminized Silk Biomaterials: A Perspective from Drug Stabilization

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ABSTRACT: Silk protein fibroins have gained remarkable attention in recent years as a potential drug carrier in the developing medicinal field of research. In this work, the stability of anticancer agent curcumin in the presence of two different silk protein fibroins from nonmulberry Antheraea mylitta (Am) and mulberry Bombyx mori (Bm) has been examined, and the possible mechanism of stabilization in a physiologically relevant medium has also been explored. In solution phase, upon treatment with curcumin, the predominated β-sheet structure of Am is marginally altered, whereas in the case of Bm, a substantial structural changeover has been observed (from coil to β-sheet) to accommodate the hydrophobic drug. Also, the morphological assessments suggest that curcumin is nicely housed in the nanoscaffold of silk fibroin (SF). Consequently, the extent of degradation of curcumin is remarkably suppressed upon encapsulation with the SF. The dissimilarity in the binding patterns of curcumin with these silk proteins could be responsible for the observed difference in the stability orders. Curcumin binds the surface of Bm, whereas in Am, the drug is incorporated in the hydrophobic cavity, and as a consequence, the drug is effectively sequestered out of the aqueous medium. The increase in the fluorescence quantum yield upon interaction with the protein greatly modulates the excited-state intermolecular hydrogen atom transfer (ESIPT) process, which is in tune with a substantial increase in the lifetime of the excited-state of curcumin. The ESIPT is known to play a crucial role in the degradation of curcumin under physiological pH conditions, which perhaps implies its potential therapeutic activity in the presence of silk. The in-depth spectroscopic analyses of curcumin–SF complexes in aqueous medium can provide useful insights for further applicative developments in bioengineering.

1. INTRODUCTION

In the past few decades, material-based drug-delivery systems have been in the forefront of biomedical research.1,2 Various biocompatible materials are under investigation to advance the therapeutic activity of drugs to achieve more effective and safer therapeutics for clinical applications.3,4 Among the various biocompatible materials reported for the fabrication of drug-delivery carriers, the silk fibroin (SF)-based biomaterials have gained remarkable interest nowadays.5 The uniqueness of the material can be with respect to its properties such as biocompatibility, self-assembling, structural properties (utilized in drug- and gene-delivery), solubility (in various solvents), and biodegradability,4 and this can be achieved by tweaking its secondary structure. Such a wide variation in the properties makes the silk protein a potent drug-delivery vehicle. Researchers are trying to improve such systems by controlling the size and structure of silk proteins fabricated into diverse morphologies.4 Recently, some researchers have suggested that silk-based microneedle devices show impressive medical applications such that the material can be used in transdermal drug-delivery.5,6

Silkworms are broadly classified, domesticated and grown, depending upon their food sources. There are two groups called mulberry (Bombyx mori; Bm) and nonmulberry (Antheraea mylitta; Am). The domesticated mulberry silkworms are commercially available throughout the world, and the nonmulberry silkworms are wild and grown commercially in tropical parts of India.7,8 This type of silk contains two varieties of proteins, namely fibrous fibroin and globular sericin.7,9 Mulberry silk is commonly known for its superior tensile strength of fibroin, and the fiber formation depends upon the β-sheet content of the silk. Actually, folding of the β-sheet reforms it into a highly ordered crystalline structure, which makes the material a potential candidate for biomedical utilization.3,10 On the other hand, the presence of some specific hydrophobic residues in nonmulberry silk caters a better mechanical property over mulberry. In fact, non-mulberry fibroin is naturally gifted with integrin-binding RGD sequences, which can bind surface active molecules and shows
substantial advantages as a biomaterial. Utilization of nanocarriers of various morphological forms of SF [three-dimensional scaffolds, hydrogel, and two-dimensional (2D) films] as in vitro drug-delivery systems is also very much promising.4,11−13

In recent years, rapid development and screening of new active pharmaceutical ingredients (APIs) are considered a remarkable improvement in science and medicine, but unfortunately, most of the drugs are poorly soluble in water and cannot reach their site of action.14 A third-generation cancer chemopreventive agent, curcumin, also shows such poor aqueous solubility like other APIs.15 Curcumin is a secondary metabolite found in the plant Curcuma longa and is one of the most active components of the Indian spice turmeric.16–18 It is the best-known diarylheptanoid consisting of two aromatic rings (aryl group) joined by the C7 chain with various substituents. In solution, curcumin exists in an equilibrium between keto- and enol-tautomers (Scheme 1), and this equilibrium plays an important role in its physicochemical properties and antioxidant activities.19,20 It has been found that curcumin has good anti-inflammatory19,21 and antioxidant properties.22 Recently, curcumin has been found to bind α-amyloid proteins that are responsible for Alzheimer’s disease.19,23 It can also be considered as a model drug for the treatment of HIV infection,24 anticystic fibrosis,25 wound-healing process, and heavy-metal toxicity in neurons.26 Actually, a lower proportion of cancer patients and world’s lowest age-correlated Alzheimer’s disease incidences are found in India, and this could be due to the usage of curcumin that has superior beneficial properties.19,25 Several studies have been conducted, and the anticancer activity of curcumin was established just a decade ago.19,25–27

Curcumin is a potential choice in the development of drugs and therapeutic agents against several diseases for its broad field of applications.19,28 Importantly, there are two major challenges which limit their targets. These are low solubility of curcumin in aqueous medium and its rapid degradation in physiological pH, especially under reducing conditions. In the basic pH condition, the free hydroxide is responsible for the degradation of curcumin. It is also rapidly degraded in the presence of light.19,25 Presence of a hydrophobic skeleton and intermolecular H-bond make curcumin very feebly soluble in aqueous medium, and that is only ~0.4 μg/mL at room temperature.27 Such a solubility is not sufficient in the biological systems for its desired activity, and moreover, this is further lowered by its rapid degradation. Hence, the development of new curcumin carriers has been a challenge in the past decade. A few methods have been developed to take care of its solubility and stability issues. For example, sodium dodecyl sulphate (SDS),28−29 cetyl trimethylammonium bromide,28−30 Triton X-100,29 reverse micelles,31 Niosomes,32 bile salt aggregates,33 and various other systems34−36 have been found to increase the solubility and stability of curcumin. Curcumin delivery by encapsulation in polymer nanoparticles,37−39 some metallo−curcumin complex,40 and a hydrogel41 have also been found to be very promising. Recently, the use of plasma and other proteins in stabilizing curcumin has also been reported,42,43 but these proteins are not efficient for a prolonged time toward curcumin stabilization. In fact, the hydrophobic interior and hydrophilic exterior are essential in stabilizing the dispersed curcumin. Here, the hydrophobic region shields curcumin from hydrolysis, and the outer hydrophilic layer stabilizes the system to remain soluble in the aqueous phase.37 Therefore, large proteins with more hydrophobic domains appear to be suitable carrier systems for curcumin and also serve the purpose of stabilization. In this prospect, β-sheet rich SF should be an appropriate choice to study the concerned issues, and more importantly, silk-based natural polymeric fibroins are strategically more useful than synthetic polymeric materials because of some of its intrinsic properties and processing requirements.11

In recent years, these silk-based materials have been developed as nanoparticles to deliver proteins, small molecules, and anticancer drugs.11 Actually, nanoparticle delivery systems are expected to improve the efficiency of various encapsulated therapeutics over the microlevel particles. Recently, some reports have been published on the modified SF media, which show controlled delivery and excellent release of curcumin from the silk scaffold moiety.44,45 Also, the mechanisms of stabilization were elucidated into three types of Bm silk films: as-cast, dried from hydrogels, and methanol-treated.46 hence, it is well-established that the transformation of Bm to nanoparticles or hydrogels can lead to a good curcumin stabilizer for controlled release applications. Therefore in this respect, it is also important to look at the specialty of the native form of Bm and the new variety Am to assess how efficient these materials are to stabilize curcumin in solution for improved delivery applications.

In this work, we investigate the solubility and stability of curcumin, mediated by silk protein fibroins, using various optical spectroscopy and morphological studies. In general, two different routes have been proposed for the therapeutic function of curcumin. One is its unique photodynamic nature in vitro where curcumin generates singlet oxygen in the excited-state. For this reason, the phototoxic characteristic of the excited-state of curcumin toward selected bacteria is possibly responsible for its versatile drug activity.49 The encapsulator-mediated stabilization and controlled release in vivo is another important aspect. Earlier photophysical reports suggest that the intramolecular hydrogen-bond-assisted intramolecular hydrogen-atom (or proton)-transfer (ESIHT or ESIPT) process plays a crucial role in drug stabilization. On the other hand, in vitro curcumin-based photodynamic therapy (PDT) investigations are also very encouraging, and recently some reports are also suggesting the high specificity of curcumin-mediated

Scheme 1. Structure of Curcumin in the Keto−Enol Forms

R

R

OH

O

O

OH

NH2

H2C

H2C

O

O

OH

R

R

OH

O

O

OH

R =

CH3

keto-enol form

di-keto form

HO

R

R

O

O

OH

CH3

O
Till date, the mechanism of phototoxicity is not clearly understood, and it is believed that ESIPT imparts a critical role in singlet oxygen generation and PDT. Therefore, an understanding of the complex behavior of ESIPT provides insight into the model for the in vivo delivery from membrane interface to endothelial cell walls and in vitro film-based PDT.

2. RESULTS AND DISCUSSION

2.1. Structural and Morphological Details of Silk Protein in the Presence of Curcumin. 2.1.1. Effect on the Fibroin Secondary Structure. Circular dichroism (CD) spectra provide detailed information about the secondary structural changes of protein during the binding process. Figure 1a,b depicts the CD spectra of the tropical nonmulberry silk protein fibroin (Am) and mulberry silk protein fibroin (Bm) with increasing concentration of curcumin. In the native form of Bm, the CD spectra show one peak around 200 nm, which generally corresponds to the coil structure, but with increasing concentration of curcumin, the peak around 200 nm disappears and a new peak appears around 218 nm, which is the suggestive peak for the \( \beta \)-sheet structure. The coil to \( \beta \)-sheet structural transformation in Bm is quite common in a hydrophobic medium such as ethanol, and the same phenomenon can also be observed by lowering of pH (from pH 7 to 5 or even lower), because this effectively induces a coil to \( \beta \)-sheet transformation in SF, which is nothing but conversion of a distorted conformation (i.e., random coil or silk I) into a more stable sheet conformation (i.e., silk II). In fact, in the presence of a hydrophobic ligand, a major conformational change is accomplished to form a stable, compact hydrophobic unit. Herein, curcumin assists in the folding of Bm, which takes place by the zipping of the backbone of the peptide chain. This transformation is quite important for its biomedical applications because many researchers have shown that the ability of the drug to be released from SF materials is governed largely by their secondary structure, especially the \( \beta \)-sheet content. In the case of Am, the \( \beta \)-sheet is regarded as the native form, and with increasing concentration of curcumin, this form stays with little change in the sheet structure.

2.1.2. Variation in the Fibroin Particle Size. We performed dynamic light scattering (DLS) measurements to understand the size distribution of SF in the presence of curcumin (Figure 2). Figure 2b indicates that under physiological pH conditions, the DLS spectrum of Bm shows four distinct peaks from the lower to upper size ranges (\( \sim 10 \) nm to 1 \( \mu m \)). However, data shows that the average size distribution of Bm is around 60 nm, which indicates that the fibroin structure is composed of a high proportion of smaller particles or low-molecular-weight species. Upon addition of curcumin, the size distribution window is shifted to the higher side with reduction of peak numbers, and the average size distributions are 114 and 116 nm due to the addition of 10 and 20 \( \mu M \) curcumin, respectively. These results indicate that in the presence of curcumin, the structure of Bm is modified from a more distributed form to a somewhat ordered structure. He et al. found the same type of structural changeover in the case of Bm, and they proposed that this is a characteristic structural conversion from the coil to \( \beta \)-sheet form. This nicely corroborates the previous results from CD study also. On the other hand, in the case of Am, the single monodispersed peak on the higher side of the size distribution window (Figure 2a) corresponds to the ordered structure (\( \beta \)-
form), and with the addition of curcumin, it transforms into a form with size distribution little bit in the higher side, indicating no further alteration of the protein structure as the β-sheet is composed of larger-sized particles. In the case of drug-delivery, the particle size is an important factor, and the useful range of particle sizes employed for medical purposes is ≤100 nm, although the larger-sized particles can absorb and carry the drug with high propensity as their surface to mass ratio is on the higher side.

2.1.3. Morphological Assessment. Field emission scanning electron microscopy (FE-SEM) study is employed to examine the morphology of SF. Figure 3a,b highlights the 2D FE-SEM image of Bm in the absence and presence of curcumin, respectively. Pure Bm shows the branched structure, and a closer look indicates that such a structure is formed via aggregation of various small nanoentities. A recent report suggested that Bm consists of polydispersed particles, and under the diffusion limited aggregation (DLA) model, a dynamic interplay between the aggregation of small and large particles is likely to be observed to form a dense architecture, whereby larger particles act as new nucleation sites for smaller particles. In the presence of curcumin, the branched aggregation of Bm loses the compactness in the architecture. The DLS data indicate that with increasing curcumin concentration, the size of Bm aggregates slightly alters its polydisperse property by converting the smaller particles into larger ones. This may be the reason behind the different morphologies of Bm in the presence of curcumin. On the other hand, the FE-SEM image of Am shows a netlike structure with certain hollow spaces (Figure S1, Supporting Information). In fact, the monodisperse particles of Am are aggregated to form a snowflake-like architecture via the regular and preferential growth, followed by some modification in the classical DLA model. In this case, at higher particle concentrations, this type of array may be set up together to form a netlike structure (highly interconnected porous structures). After addition of curcumin, no change in the morphology of Am is observed, and this supports the previous results. A detailed scrutiny of the morphology could provide the location and encapsulation states of curcumin in these materials. The porous structure of Am permits curcumin to adhere within the scaffold and provides the maximum area around the encapsulated drug. Whereas in case of Bm, curcumin can manage to attach to the surface or at the interfacial position of the dense architecture of Bm.

2.2. Silk Media Assists Curcumin Solubilization. The solubility of curcumin can be followed by steady-state ultraviolet–visible (UV–vis) absorption as a function of the concentration of Am and Bm (Figure 4a,b). Curcumin has very low solubility in aqueous neutral phosphate buffer solution, but with increasing protein concentration, solubilization of curcumin is substantially facilitated. In aqueous buffers, the absorption spectra of curcumin exhibit a characteristic broad peak at 430 nm and a small shoulder at 355 nm, which are assigned to the lowest (π−π*) transition of the conjugated curcumin and feruloyl unit, respectively. With the addition of Bm, the absorbance of curcumin increases without affecting the spectral pattern. On the other hand, with increasing concentration of Am, the broad peak around 430 nm becomes prominent with a slight blue shift (by ~4 nm), and the shoulder peak at 355 nm disappears with the appearance of a new shoulder at 450 nm. These indicate that curcumin partitions from the aqueous phase into the nonpolar-like environment, very similar to that in chloroform. The spectrum displays vibronic structures with absorbance maxima near 420 nm, and this corresponds to the highly conjugated protonated enol. The absence of the 450 nm shoulder structure and the quite-aqueous-like spectral pattern of curcumin in the presence of Bm.
suggests that this drug molecule has adsorbed on the surface of the protein where aqueous phase is in close proximity.

2.3. Excited-State Properties of Curcumin and Delineation of the Binding Location. To interpret the binding interaction between curcumin and SF, we monitored the change in the fluorescence of curcumin with the addition of silk protein (highlighted in Figure 5a,b). Under physiological pH conditions, the quantum yield of curcumin is very low, and it can be regarded as almost nonfluorescent. However, the addition of the silk protein to the buffered curcumin leads to a substantial enhancement with a blue shift in the emission profile, and it reaches saturation above certain concentration. This indicates that the fibroin protein facilitates solubilization through partitioning of curcumin molecule from the bulk aqueous to proteinous medium. The quantum yield (Φ) of curcumin is also found to vary with the protein concentration and reaches a limiting value (Figure 5c). The observed limiting quantum yield is 0.062 and 0.020 for Am and Bm, respectively. Interestingly, the increase in the quantum efficiency of curcumin in two different fibroin media is quite different, which is possibly because of the differences in the location of binding. The fluorescence quantum yield of curcumin is predominantly controlled by the perturbation in the ESIPT process.32 ESIPT is considered to be the primary factor responsible for the reduction in the fluorescence efficiency of curcumin, and any perturbation on this imparts a larger influence on the enhancement of fluorescence intensity via blocking of the nonradiative decay processes, and this will be discussed later on. Therefore, with the addition of SF, the ESIPT of the drug is largely affected because of the change in the surrounding environment of the molecule. The comparatively low Φ in Bm suggests that the ESIPT process is still in operation, whereas in the presence of Am, a large modulation of the ESIPT process results in higher Φ. Therefore, this result indicates that the curcumin molecule is attached to the more solvent (aqueous) assessable part of Bm.

We employed steady-state anisotropy (r0) measurements and micropolarity analysis of the bound drug to find out the location of curcumin in the protein medium. The value of anisotropy at the saturation point is maximum in the case of Am than that in Bm (0.323 and 0.318 for Am and Bm) (Figure S2, Supporting Information), which clearly indicates that the drug molecule is tightly incorporated in the rigid environment of Am than in Bm. In line with the previous analysis, a substantial rigidity in the microenvironment of the Bm−curcumin complex arises because of the stronger surface occupation of the drug, which is presumably due to the interfacial adsorption. In the micropolarity analysis, at a maximum concentration of SF, the E_r(30) values are around 43.52 and 45.29 for Am and Bm, respectively [from E_r(30) scale presented in Table S1, Supporting Information]. These values suggest that the probe is located in a more hydrophobic region in Am compared to that in Bm environment. Because both the fibroins are rich in the β-sheet structure, this little difference in micropolarity after binding with curcumin indicates that drug is positioned slightly away from the hydrophobic core of Bm, which perhaps signifies the surface attachment, as also evident from the previous results.

2.4. Interpretation of the Binding Interactions and Thermodynamics Details. So far, we have found that the fibroin protein can serve as an excellent protector of curcumin from the external aqueous environment. Actually, the nature of interaction between the drug and fibroin can assess on how efficient these proteins can be as carrier of curcumin. To find out the binding stoichiometry of curcumin with SF, we employed the Job’s plot analysis (Figure S3, Supporting Information).

Figure 5. Fluorescence emission spectra of curcumin (10 μM) as a function of concentration of (a) SF Am and (b) SF Bm. (c) Variation of the quantum yield (Φ) of curcumin with concentration of Am or Bm (inset). Symbols with vertical cap indicate the error bar.
Information) using the fluorescence technique.54 The results suggest that the fibroin protein forms 1:1 stoichiometric complex with curcumin (n = 1 for Am and n = 1.2 for Bm). Since the binding stoichiometry is found to be close to unity, we have determined the binding constant for curcumin − fibroin complexation by employing the Benesi−Hildebrand (B−H) equation.55 The estimated binding constant (K_{BH}) of curcumin with Am and Bm are (3.87 ± 0.15) × 10^5 and (1.65 ± 0.06) × 10^6 M^{-1}, respectively, at pH 7.4 at 298 K. The value of K_{BH} was obtained from the slope of (I_\infty − I_0)/(I_t − I_0) versus [protein]^{-1} plot, (B−H plot) (Figure S4, Supporting Information and Table 1). The results indicate that the values are on the order of 10^5 to 10^6, which infer quite a strong complexation. The binding efficiency of curcumin is much higher with Bm than with Am. This can be explained by the binding-induced structural transition of Bm from the random coil to a more ordered β-sheet structure. This further indicates that the curcumin-mediated structural transition of Bm results in a greater binding strength. As hydrophobic interactions are the dominant driving forces for the β-sheet folding in aqueous medium, curcumin possibly reinforces the hydrophobic interaction between the two peptide chains of Bm.56 In the protein segment, various other forces are still in operation, and among them, H-bonding is always an integral part of the interaction.

The possibility of hydrogen bond formation with curcumin is strongly dependent on the ionic strength of the medium.57,58 Actually, salt is regarded as a good substance for breaking and making of H-bonds, thereby promoting the formation of intermolecular H-bonding of curcumin, and resulting in the modulation of the ESIPT rate.58 Here, with the addition of salt (NaCl) in the curcumin−fibroin complex, the fluorescence intensity is found to be enhanced with a considerable blue shift of the emission maxima (Figure 6a,b). However, no noticeable change in the absorption spectra was found, which means the addition of salt affects the excited-state predominantly, thereby modulating the ESIPT rate. We could not monitor the ESIPT at the higher salinity range because of the instability of SF. As

| system | T (K) | K_{BH} (M^{-1}) | R^2 | ΔH^0 (kJ mol^{-1}) | ΔS^0 (J mol^{-1} K^{-1}) | ΔG^0 (kJ mol^{-1}) | R^2 |
|--------|------|----------------|-----|----------------------|--------------------------|----------------------|-----|
| Am     | 288  | (4.97 ± 0.14) × 10^5 | 0.985 | −11.57 ± 0.09 | 68.51 ± 0.10 | −31.40 ± 0.12 | 0.939 |
|        | 298  | (3.87 ± 0.15) × 10^5 | 0.983 | −31.87 ± 0.18 | 32.64 ± 0.09 | −33.45 ± 0.10 | 0.917 |
|        | 308  | (3.44 ± 0.08) × 10^5 | 0.996 | −34.63 ± 0.14 | 36.48 ± 0.07 | −37.45 ± 0.09 | 0.938 |
|        | 318  | (3.12 ± 0.12) × 10^5 | 0.989 | −34.63 ± 0.14 | 36.48 ± 0.07 | −37.45 ± 0.09 | 0.938 |
| Bm     | 288  | (1.91 ± 0.08) × 10^6 | 0.991 | −7.15 ± 0.18 | 95.02 ± 0.21 | −35.47 ± 0.12 | 0.938 |
|        | 298  | (1.65 ± 0.06) × 10^6 | 0.992 | −7.15 ± 0.18 | 95.02 ± 0.21 | −35.47 ± 0.12 | 0.938 |
|        | 308  | (1.54 ± 0.03) × 10^6 | 0.999 | −7.15 ± 0.18 | 95.02 ± 0.21 | −35.47 ± 0.12 | 0.938 |
|        | 318  | (1.42 ± 0.04) × 10^6 | 0.998 | −7.15 ± 0.18 | 95.02 ± 0.21 | −35.47 ± 0.12 | 0.938 |

Figure 6. Fluorescence emission spectra of curcumin (10 μM) with the addition of NaCl in (a) silk Am and (b) silk Bm. (c) Emission spectra of the curcumin−Am complex as a function of temperature. (d) van’t Hoff plot for the binding of curcumin with SFs in pH 7.4.
the salt concentration increases, the fibroins are finally spun into water-insoluble fibers by mechanical shear and the stretching action of the spinneret.59,60 The addition of salt also enhances the interaction of curcumin with protein segments to perturb the H-bonding with the fluorophore and effectively reduces the ES I PT-mediated nonradiative processes.

Electrostatic interaction, hydrophobic force, van der Waals force, and H-bonding interaction are the major forces operating in chemical and biological systems.48 Ross and Subramanian presented a thumb rule to explicate the nature of the binding interaction, depending on the signs and magnitudes of thermodynamic parameters ($\Delta H^0$ and $\Delta S^0$).49 At first, we varied the temperature at some fixed composition of Am–curcumin (Figure 6c), and we observed that with increasing temperature, the fluorescence intensity decreases. This means that at elevated temperatures, the equilibrium between the bound and unbound forms of curcumin with the silk protein gets disturbed, and some fraction of curcumin is thrown out bound and unbound forms of curcumin with the silk protein that at elevated temperatures, the equilibrium between the

![Image](https://acsof.org/article/6761)

**Figure 7.** Extent of degradation of curcumin (10 $\mu$M) in terms of the decrease in absorbance maxima with increasing time interval.

pseudo-zero-order kinetics, the observed degradation rates of curcumin are around $3.3 \times 10^{-3}$ and $1.7 \times 10^{-3}$% min$^{-1}$ in the presence of Am and Bm, respectively. This indicates that curcumin is well-fibroinized to get better stability than the other reported protein-containing environment, for example, human serum albumin (0.20% min$^{-1}$), fibrinogen (0.22% min$^{-1}$), transferrin (1.80 and 0.18% min$^{-1}$), and immunoglobulin G (2.92 and 0.58% min$^{-1}$).49,50 Also cyclodextrins can provide quite a similar kind of degradation rate.51 Previous reports show that the stability of curcumin is greatly enhanced in micelle, niosome, vesicle, and polymeric media.14,28,32,62,63 Some recent reports proposed that polymeric nanoparticles can also be efficient to stabilize curcumin for a prolonged time.37,38 But in the case of synthesized carrier systems, toxicity is always a matter of concern. Therefore, on the issues of biocompatibility and biodegradability, the natural proteins should be preferable for drug carrying and stability.2,64 As a comparison, the stability of curcumin is found to be higher in Am than in Bm. Nowadays, Bm is frequently used as a drug-delivery system, and similar systems as a carrier protein.

2.5. Stability of Curcumin in Silk Fibroins: Comparison with Other Media. In aqueous medium, the solubility of curcumin is substantially altered with change in pH of the medium from acidic to alkaline. However, in alkaline or neutral pH, curcumin undergoes rapid degradation, and it is due to the instability of the $\beta$-diketone linker, which rapidly dissociates to vanillin, ferulic acid, and feruloyl methane.52 In aqueous buffer (pH 7.4) solution, almost half the proportion of curcumin was degraded in around 30 min of interval.37,61 In the previous section, we found that in the presence of these SFs, the solubility of curcumin was greatly enhanced. Therefore, an estimation of the extent degradation is required to assess the stability. The degradation rates of curcumin in the presence of Am or Bm were investigated using the UV–vis absorbance method, and the changes are highlighted in Figure 7. In the

**Table 1.** Magnitudes of thermodynamic parameters ($\Delta H^0$, $\Delta S^0$, $\Delta G^0$) for curcumin–SF complexes, estimated by using eq 2 at different temperatures, are shown in Table 1 also. The negative value of the standard Gibbs free energy change ($\Delta G^\circ$) indicates the spontaneous nature of the interaction between the fibroin protein and curcumin. The $\Delta G^\circ$ [(−31.87 ± 0.18) and (−35.47 ± 0.12) kJ/mol for Am and Bm, respectively, at 298 K] values are mostly guided by the negative values of $\Delta H^0$ [(−11.57 ± 0.09) and (−7.15 ± 0.18) kJ/mol for Am and Bm, respectively] and positive values of $\Delta S^0$ [(68.51 ± 0.10) and (95.02 ± 0.21) J/K mol for Am and Bm, respectively]. Hence, the thermodynamics of the binding of curcumin with SF is strongly assisted by the exothermicity as well as positive entropy. A very low buffer solubility and a substantially higher solubilization of curcumin in silk protein signify that the hydrophobic force of interaction could be the major binding force in the association process. According to Ross et al.,59 the sign of $\Delta H^0$ and $\Delta S^0$ would be positive for the hydrophobic effect. In low temperatures, the positive $\Delta S^0$ (hydrophobic effect) is the main contributor for the spontaneous association. However, we have estimated negative and positive values for $\Delta H^0$ and $\Delta S^0$, respectively. Therefore, the force responsible for a large negative contribution of $\Delta H^0$ compensates the hydrophobic force (positive $\Delta H^0$) effectively, and these would be the van der Waals force, hydrogen-bonding, and ionic interaction (relatively less contribution). Therefore, from the estimated magnitude and sign of the thermodynamic parameters (Table 1), we can conclude that the hydrophobic interaction is predominant in the case of Bm–curcumin association, whereas the other forces are largely operative in the Am–curcumin association.

2.6. Role of the Excited-State Processes from the Perspective of Drug Stabilization. As mentioned earlier, the unique phototoxic nature of curcumin comes from the excited-state dynamic phenomena so that the long-lived excited-state
Figure 8. Time-resolved lifetime decays of curcumin (10 μM) in the SF medium: (a) Am (from 0 to 5 μM) and (b) Bm (from 0 to 1 μM).

Table 2. Fluorescence Lifetime (λex = 442.6 nm), Radiative and Nonradiative Parameters of Curcumin in Different Concentrations of Am and Bm at T = 298 K

| Concentration (μM) | τ1 (ns) | a1 | τ2 (ns) | a2 | τΦ (ns) | Φ | k1 × 108 (s−1) | k2 × 109 (s−1) |
|-------------------|---------|----|---------|----|---------|----|----------------|----------------|
| Am Fibroin        |         |    |         |    |         |    |                |                |
| 0.125 μM          | 0.153   | 0.97 | 0.712   | 0.03| 0.170   | 0.0058| 0.34           | 5.85           |
| 0.5 μM            | 0.229   | 0.88 | 0.772   | 0.12| 0.294   | 0.0148| 0.50           | 3.35           |
| 1.25 μM           | 0.277   | 0.83 | 0.817   | 0.17| 0.369   | 0.0390| 1.06           | 2.60           |
| 2.5 μM            | 0.305   | 0.79 | 0.839   | 0.21| 0.417   | 0.0519| 1.24           | 2.27           |
| 5 μM              | 0.334   | 0.78 | 0.861   | 0.22| 0.450   | 0.0622| 1.38           | 2.08           |
| Bm Fibroin        |         |    |         |    |         |    |                |                |
| 0.025 μM          | 0.122   | 0.98 | 0.723   | 0.02| 0.134   | 0.0025| 0.19           | 7.44           |
| 0.1 μM            | 0.161   | 0.96 | 0.881   | 0.04| 0.190   | 0.0051| 0.27           | 5.24           |
| 0.25 μM           | 0.232   | 0.92 | 0.994   | 0.08| 0.293   | 0.0098| 0.35           | 3.38           |
| 0.5 μM            | 0.305   | 0.90 | 1.002   | 0.10| 0.375   | 0.0148| 0.40           | 2.63           |
| 1 μM              | 0.334   | 0.89 | 1.075   | 0.11| 0.416   | 0.0199| 0.48           | 2.36           |

Experimental errors in the determination of lifetime: ±5%.

species will have substantial importance in the therapeutic activity. It has been found that in various organized media, the excited-state lifetime of curcumin is considerably increased, and it is considered that these media have the role of stabilizing curcumin that is necessary for increased therapeutic activity. Therefore, an understanding of the complex excited dynamics of curcumin in solution and in organized media is very much crucial in fabricating or selecting some effective carrier encapsulates. Excited-state photophysics of curcumin primarily depends on the ESIPT and solvation processes. ESIPT is considered as one of the major factors for deactivation of the excited-state through nonradiative processes, leading to shortening of the fluorescence lifetimes. There are some reports on the excited-state behavior of curcumin in neat and mixed solvents. In nonpolar solvents, the proton transfer (PT) process is primarily guided by formation of the intermolecular six-membered hydrogen-bonded chelate ring of the cis-enol form. Such a PT is very fast and completed within a few hundreds of femtoseconds. However, in polar protic solvents such as methanol, the intramolecular H-bonding gets perturbed because of the influence of the solvent molecule with the H bond of curcumin. Saini and Das investigated the excited-state dynamics of curcumin in the presence of various mixed solvents, and they found that variable properties, for example, viscosity, polarity, and hydrogen-bonding ability, of the alcohol solvent have key roles in the excited-state processes in the toluene–alcohol mixtures. Leung et al. showed by the fluorescence upconversion technique that curcumin encapsulated in polyester nanoparticles exhibit three types of decay processes, where the very fast one (∼2–4 ps) is assigned to the reorganization of water molecules, time constant of ∼50 ps is responsible for the ESIPT of curcumin, and a slow decay component 200–400 ps is also present. Adhikary et al. reported that the ESIPT process is primarily observed in the micelle-encapsulated curcumin molecule, where deuteration of curcumin has a negligible effect on the fast component, and the second component (responsible for ESIPT, τ ≈ 50 ps) exhibits a pronounced isotope effect. Recent reports demonstrated that in the presence of various organized media, the ESIPT of curcumin is greatly modulated with a decrease in the contribution of fast ESIPT processes to slow decay processes followed by an increase in the fluorescence lifetime. The fluorescence decays of curcumin were measured in the presence of SFs with an excitation wavelength of 442.6 nm, and the fluorescence decays were monitored in the respective emission maxima. Figure 8 depicts the fluorescence decay pattern of curcumin with increasing concentration of SF. The lifetime values, obtained by fitting these decay plots with the biexponential decay model, are tabulated in Table 2. In some reports, the fast component in the biexponential lifetime has been considered responsible for ESIPT, although on the picosecond time scale, it would be very hard to identify and explicate the exact processes. Therefore, we estimated the average lifetime of curcumin by the following eq 4 for simpler explanations because in a heterogeneous protein pocket, curcumin experiences different type of interactions, resulting in a complex decay process. From Table 2, it is evident that the average lifetime of curcumin sharply increases with increasing SF concentration. At some optimum concentration of SFs (5 μM for Am and 1 μM for
Bm), the average lifetime value of curcumin is found to be 0.450 and 0.416 ns for Am and Bm, respectively, with individual components 0.334 ns (0.78%) and 0.861 ns (0.22%) for Am and 0.334 ns (0.89%) and 1.075 ns (0.11%) for Bm. The average fluorescence lifetime of curcumin in these protein solutions is found to be very close to those in octanol and in a composed vesicular system, that is, the bilayer region of the small unilamellar niosomes. This indicates that microenvironments surrounding the drug molecules are quite alike. The lifetime of curcumin in the presence of serum protein is also well-modified \((\tau_{av} \approx 217 \text{ ps})\), but in comparison with the silk protein, this value is quite less. This means that the silk protein protects curcumin very well and it also provides a better stability. The obtained average lifetime value inside Bm is less compared to Am, which clearly indicates that the modulation of the ESIPT process of curcumin occurs because of the different drug–protein binding characteristic, primarily related to the location of curcumin inside the protein cavity. Importantly, because of the different structures of Am and Bm, the location of attachment of the drug is naturally different, and the solvated protein water can play some important roles to perturb the ESIPT process of curcumin, leading to different lifetime values. In particular, the strength of the intermolecular hydrogen-bonding between curcumin and the surrounding molecules also affects the ESIPT. In this case, curcumin is capable of forming hydrogen bonds with the peptide and disulfide linkage, resembling the protic nature of the surrounding medium. The substantial increase in the average lifetime \((\tau_{av} \approx 0.450 \text{ ns} \text{ for Am and 0.416 ns for Bm})\) values indicates the stability of drugs for controlled drug-delivery. Also, with an increase in the salinity in the drug–protein cocktail, the ESIPT is found to be remarkably modulated, which perhaps implies the involvement of H-bonding in the keto–enol moiety with the silk protein. Eventually, this study paves an excellent route to stabilize curcumin in solution (in physiological pH condition) and subsequent drug activity. The natural biocompatible SF (Bm and especially Am) can be employed as promising drug stabilizers for curcumin and other related drugs for controlled drug-delivery.

4. MATERIALS AND METHODS

4.1. Materials. Curcumin (HiMedia, India) was purified by the column chromatographic technique, and the purity of the sample used was \(\geq 99\%\) (checked by high-performance liquid chromatography). NaCl (analytical grade) was purchased from Merck, India and ethanol (UV spectroscopic grade) was purchased from Spectrochem, India. All other chemicals purchased were of analytical grade. Ultrapure Milli Q water was used in the study. All solutions were prepared in 5 mM sodium phosphate buffer of pH 7.4 (±0.1). The pH value was measured with a precalibrated Eutech pH 510 ion pH-meter.

4.2. Preparation of Silk Protein Fibroin Solution. Nonmulberry tropical Tasar (A. mylitta, Am) 5th instar silk larvae were collected from our biotechnology farm, IIT Kharagpur, West Bengal, India. Fibroin was isolated following the previously reported method.53 In brief, silk glands were collected by sacrificing the larvae. The fibroin was collected from the gland by squeezing and storing at 4°C for immediate use or at \(-20\)°C until use. The fibroin was dissolved in the aqueous buffer containing 1% \((w/v)\) SDS, 10 mM Tris (pH 8.0), and 5 mM ethylenediaminetetraacetic acid. The solution was centrifuged at 5000 rpm for 10 min, followed by the dialysis of the supernatant against ultrapure water for 6–8 h in a 12 kDa dialysis membrane (Sigma, USA). The fibroin was concentrated in a 3.5 kDa dialysis membrane (Thermo Fisher, USA) against 30% poly(ethylene glycol) PEG 6000 solution.

The fibroin from mulberry B. mori (Bm) was isolated from cocoons following the reported protocol. Briefly, cocoon pieces were degummed in 0.02 M Na2CO3 solution for 1 h and washed thoroughly with distilled water. The degummed fibroin fibers were dissolved in 9.3 M aqueous LiBr solution and incubated for 4 h at 60°C. The solution was dialyzed [molecular weight cutoff (MWCO) 12 kDa] against distilled water for 48–72 h. The fibroin concentration was determined by measuring the residual mass after drying at 37°C.

4.3. Preparation of Solution. A stock solution of curcumin was prepared in methanol, and then methanol was evaporated. To this, the required volume of pH 7.4 (±0.1) phosphate buffer along with the required amount of fibroin solution was added to get the final concentration of curcumin.
samples and the mixture was kept for an hour at room temperature. The pH value of the prepared Bm SF solution was in the range of 6.5–7.0, which is similar to the reported value. The pH value of the Am SF solution was in the range of 7.2–7.5. The fibroin solution having pH in this range was used in sodium phosphate buffer of pH 7.4 (±0.1) for preparing the fibroin–curcumin mixed solution throughout the experiment. All other reagents for this study were dissolved in Milli-Q water.

4.4. Instrumentation and Methods. The UV–vis absorption spectra were recorded on a Shimadzu UV-2450 absorption spectrophotometer by scanning the solution in the wavelength range of 250–600 nm. CD spectra were recorded on a Jasco-815 automatic recording spectropolarimeter at 298 K over a wavelength range of 190–270 nm, with a scan speed 50 nm/min under constant N2 purging. A quartz cell having a path length of 0.1 cm was used, and three successive scans were accumulated for each spectrum. The baseline was corrected by an appropriate buffer solution running under the same conditions (blank) and subtracted from the experimental spectra.

DLS measurements were attempted using a Malvern Nano ZS instrument (model no. ZEN3600) having a thermostated sample chamber. All measurements were carried out using a 4 mW He–Ne laser (λ = 632 nm).

FE-SEM images were collected using a FEI NOVA NANOSEM 450 instrument working at 5 KV. The film samples were spread on a glass slide and coated with gold particles in a sputter coater. The steady-state fluorescence spectra were recorded on 298 K on a Horiba Jobin Yvon spectrophotometer (Fluorolog-3) equipped with a water-cooled temperature-controlled cuvette holder, and a quartz cuvette of 1 cm path length was used. The samples were excited at 420 nm, and the emission wavelengths were recorded from 430 to 700 nm with different slit widths (2/1 nm for Am and 3/2 nm for Bm).

Steady-state anisotropy (r) measurements were also carried out with the Fluorolog-3 spectrophotometer. The steady-state anisotropy (r) is expressed as follows

\[ r = \frac{(I_{VV} - GI_{VH})}{(I_{VV} + 2GI_{VH})} \]  

(5)

\[ G = \frac{I_{VV}}{I_{VH}} \]  

(6)

where \( I_{VV} \) and \( I_{VH} \) are emission intensities collected from the sample when the excitation polarizer is oriented vertically and the emission polarizer is placed vertically and horizontally, respectively. \( G \) is the correction factor for the instrument and is estimated by keeping the excitation polarizer horizontal and emission polarizer vertical and horizontal, respectively.

The fluorescence quantum yield (Φ) was determined using C153 in acetonitrile (as a secondary standard probe with \( \Phi = 0.56 \)) and \( \Phi \) of curcumin was estimated by the following equation

\[ \Phi_S = \frac{A_S}{A_R} \times \frac{(Abs)_R}{(Abs)_S} \times \frac{n_S^2}{n_R^2} \]  

(7)

Here, ‘A’ terms denote the area under the fluorescence curve; Abs denotes absorbance; \( n \) is the refractive index of the medium; \( \Phi \) is the fluorescence quantum yield; and subscripts S and R denote the parameters for the studied sample and reference, respectively.

The fluorescence lifetime was measured using a time-correlated single photon counting spectrometer from Edinburgh Instrument Ltd. (U.K.). The samples were excited at 442.6 (±10) nm using a picosecond laser diode (EPL-445 pulse width ~67.9 ps), and signals were collected at a magic angle of 54.7° with a high-speed photomultiplier tube (H10720-01, photosensor module from Hamamatsu, Japan). The fluorescence decays were monitored at the corresponding emission maxima, as observed in the steady-state fluorescence measurement. The instrument response function of our setup is ~230 ps. The data were analyzed using FAST decay analysis software from Edinburgh Instruments. All fluorescence decays were fitted with a biexponential function, considering a \( \chi^2 \) value close to 1, which is an indication of good fitting.

The average lifetimes of curcumin, after fitting the fluorescence transients with a biexponential function, were estimated by using the equation

\[ \tau_{av} = a_1\tau_1 + a_2\tau_2 \]  

(8)

where \( \tau_1 \) and \( \tau_2 \) are the first and second lifetime components, respectively, which were monitored at the emission maxima of the fluorophore and \( a_1 \) and \( a_2 \) are the respective amplitudes of these components.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b00809.

FE-SEM images of Am (1 mg mL\(^{-1}\)) and Bm (1 mg mL\(^{-1}\)) in the presence of curcumin (50 μM), change in the steady-state fluorescence anisotropy of curcumin with increasing concentrations of Am and Bm, Job’s plot for the the curcumin–SF complex, B–H plot for the complexation of curcumin with Am and Bm, and \( E_S(30) \) values versus Stokes shifts of curcumin with variation of SF concentration (PDF)

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Author Contributions

S.P. prepared all solutions for spectroscopic studies; collected, analyzed and interpreted data; and wrote parts of the manuscript. S.B. extracted and prepared protein samples from a natural source. S.C.K. supplied the silk protein samples and helped to evaluate, edit, and revise the manuscript. M.H. planned the project, supervised the development of work, and finalized the manuscript.

Notes

The authors declare no competing financial interest.
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