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Nanocavity Effect On Photophysical Properties Of Colchicine: A Proof by Circular Dichroism Study and Picosecond Time-Resolved Analysis in Various Reverse Micellar Assemblies

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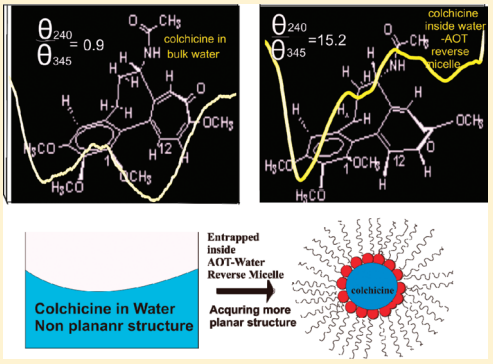
Supporting Information

ABSTRACT: In August 2009, colchicine won Food and Drug Administration (FDA) approval in the United States as a stand-alone drug for the treatment of acute flares of gout and familial Mediterranean fever. Recently, it is now the center of attraction in medicinal research. In this present paper, we have employed two other analogues of colchicine for exploring the photophysical properties inside nanocavity environment in details. Here we have a series of interesting results that have interesting similarity with the colchinoid−tubulin interaction. To monitor fluorescence properties of colchinoids, we have used absorption, emission, and time-resolved spectroscopy and to monitor structural properties we have measured circular dichroism. Steady-state anisotropy and dynamic light scattering results give an idea about the microenvironment sensed by the colchinoids molecules. A sharp increment for colchicine, very small increment for isocolchicine and no increment for colcemid in fluorescence and different circular dichroism (CD) spectra of all of these colchinoids upon embedment inside nanocavity of reverse micelle made a supposition that all these changes of fluorescence properties and CD results of colchinoids is not solely due to viscosity effect but also the constraint, that is, very narrow space to spread over, given by the nanocavity of reverse micelle. Moreover, we have noticed that the B ring of the colchinoids also have a pronounced effect on the interaction nature as well as on conformational change of these compounds after entrapment.



1. INTRODUCTION

Colchicine, a toxic natural product and secondary metabolite, was originally extracted from dried seeds of plants of the genus Colchicum (Autumn crocus, *Colchicum autumnale*, also known as "Meadow saffron") and *Gloriosa superba*.[1] Colchicine, chemically known as *N*-(5,6,7,9-tetrahydro-1,2,3,10-tetramethoxy-9-oxo-benzo[a]heptalen-7-yl) acetamide,[2] was first isolated in 1820 by the two French chemists P. S. Pelletier and J. Caventon[3] and its structure is now well-known.[4−9] Interest in the compound colchicine has been markedly increased in recent years by the discovery that it arrests the process of cell division (mitosis) in plant or animal cells.[10] In August 2009, colchicine won Food and Drug Administration (FDA) approval in the United States as a stand-alone drug for the treatment of acute flares of gout and familial Mediterranean fever.[11] In experimental studies, it has been used as a neurotoxin in animal models of Alzheimer's disease and epilepsy.[12−16] In the last decades, colchicine has received considerable attention in basic cancer research because it disrupts mitosis, ending the process at the metaphase.[17−20] Actually, colchicine has been widely used as probe to understand the role of microtubules in cells due to its high affinity for tubulin, where a so-called colchicine binding site is defined.[21−25] Ulusu et al. have conducted a comperative study with colchicine on glutathione reductase to understand the drug potency of this enzyme.[26] That is why this drug molecule became very crucial in medicinal chemistry and a large amount of work has been done on interaction of colchicine with various proteins[27a,b] especially with tubulin. Some authors have found that colchicine triplet excited states can be used as reporters for the protein binding sites, as their lifetimes are highly sensitive to the supramolecular microenvironment experienced within these biomacromolecules.[28−31] In this article, we have used two other analogues of colchicine in which one of them isocolchicine, a structurally similar analogue but does not bind with tubulin, and the other is active to tubulin but does not fluoresce upon binding. In this work, we have investigated the photophysical properties of colchicine and its analogues inside a reverse micelle.

It was already reported that although colchicine has no intrinsic fluorescence property in any type of bulk solvent it gives highly enhanced fluorescence when it binds with tubulin.[32] The fluorescence can be ascribed to the tropolone moiety (C ring) of the drug and has the properties of a $\pi-\pi^*$ transition with fluorescence lifetime (1.14 ns) characteristic of the singlet state[32−34] and upon this binding quantum yield of colchicine become 0.03.[32] This fact was demonstrated by the introduction of

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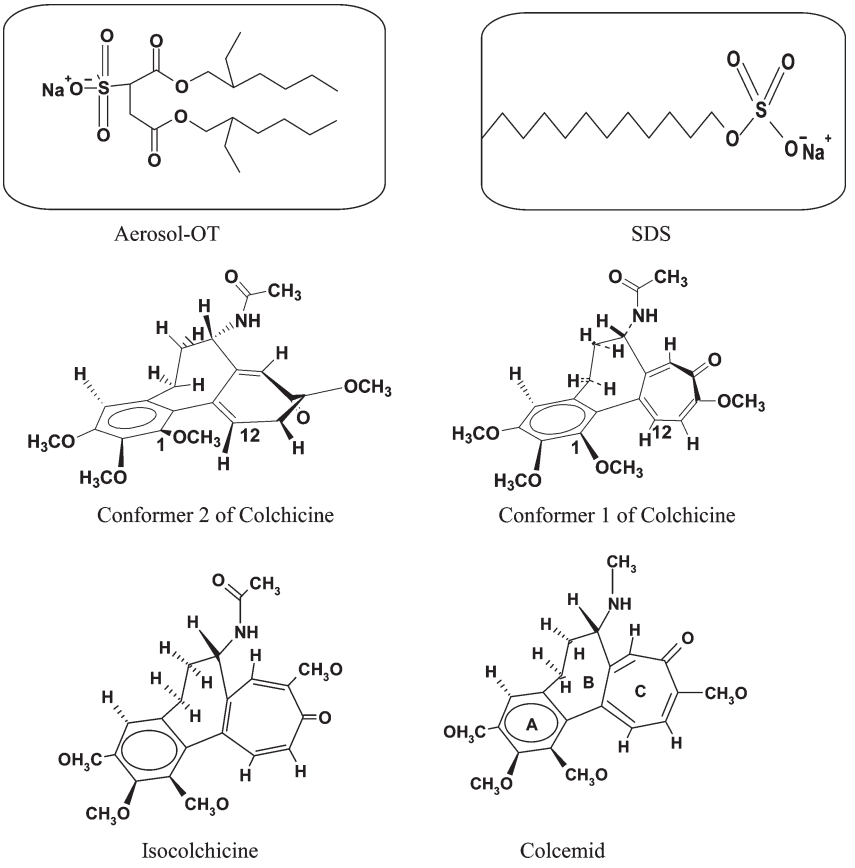
Scheme 1



hydrophobic environment given by protein moiety as well as a probable suggestion that a conformational change occurs from boatlike conformer (1) to more planar conformer (2).[32] There are several instances of immobilized fluorescence, for example, reduced flavines show appreciable fluorescence upon binding with protein.[35] The viscosity- or binding-dependent fluorescence enhancement shown by auramine O[36] has mentioned and has been interpreted by Oster and Nishijima as resulting from a restriction of internal rotation of the aromatic groups with respect to one another resulting from highly viscous media or the binding to the surface of macromolecule.[37] It is of interest that the flexible, triphenylmethane dye does not fluoresce in water or organic solvents but does fluoresce in glycerol or when bound to methacrylate polymers due to diminished internal conversion reactions as internal rotation becomes restricted.[38] The small viscosity effect on the fluorescence of the rigid molecule anthracene, compared to the large viscosity effect on the fluorescence of di-9-anthryl ethane, constitutes another the case of immobilization of a flexible molecule.[39] So in all cases we can see that there are the following two main reasons for enhancement of otherwise non-fluorescent flexible biological compounds: viscosity, given by bulk solvent, and restriction, given by attachment with protein or polymer. Here, we choose colchicine as a drug and sodium dodecyl sulfate (SDS) and sodium 1,4-bis(2-ethylhexyl)sulfosuccinate (AOT) surfactant as a long chain molecule and try to observe if any such increment of fluorescence occurs inside reverse micellar core or not. In this study, we have used aqueous as well as non-aqueous reverse micelle to compare this enhancement of fluorescence of colchicine molecule.

Natural colchicine has a stereogenic atom C (7) and an axis of chirality, as the benzenoid A-ring and the troponoilic C-ring are twisted 53° with respect to each other. Colchicine is optically active and exhibits a double-negative Cotton effect in its optical rotatory dispersion spectrum;[40] one occurs at about 330 nm from the methoxytropone system, while the second at 280 nm has been assigned to the K-band of the biaryl system. It was reported that the negative circular dichroism (CD) band of the colchicine at 340 nm either vanishes or is greatly reduced in magnitude when it is bound to tubulin, suggesting either that the colchicine binding site on tubulin cancels the inherent asymmetry of the colchicine or that a conformational change in the colchicine molecule accompanies the binding of colchicine to tubulin.[41] In our study, we also want to examine whether there is any type of conformational change occurring in colchicine molecule when it is encapsulated in the nanocavity of reverse micelle. To examine this steriochemical change we have taken two other analogues of colchicine inside a series of different AOT reverse micelle and also a SDS reverse micelle that are mimicking the primary requirement for fluorescence of colchicine.

Reverse micelles can be schematically represented as globular aggregates constituted by an internal core made up by surfactant hydrophilic heads and an external region formed by the surfactant alkyl chains, and these can share many fundamental properties of biomembranes such as the dominance of interfacial effects on their behavior and the existence of an ordered array of oriented molecules making them well-suited to act as membrane models.[42]

In particular, colchicine is our interested compound for this purpose but to follow the results precisely we have taken the

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analogues that have differences in their structure. Here, we have studied emission spectrum of all the colchinoids inside several types of AOT-reverse micelle by using different core solvent such as water, ethylene glycol, methanol, and dimethylformamide (DMF) and try to follow the trend of fluorescence intensity with increase in W_0 values of any particular reverse micelle. The fundamental aim in this present article is very simple reflecting the nature of photophysical properties of colchicine in restricted environment having higher viscosity as well as constraint over the colchicine molecule. Also, we have tried to explore a comparable idea about the immobilized fluorescence observed in many heterofores, such as reduced flavine, auramine O, diarylalkanes, and so forth, which have molecular framework just like our colchinoids. Emphasis has been given on a particular fact that whether there is any enhancement of fluorescence intensity of colchinoids after entrapment inside aqueous as well as nonaqueous reverse micelle and what are the main factors responsible for this enhancement between the constraints due to the nanocavity or the higher microviscosity of the nanocavity region or any other factors.

2. EXPERIMENTAL SECTION

Colchicine was taken from SRL (99%) and colcemid and Aerosol-OT were taken from Sigma-Aldrich and used as received. AOT was dried over 36 h with a high-energy vacuum pump. Isocolchicine has been synthesized accordingly by previous report.[43] All the solvents used were purchased from Sigma Chemicals and used as-received. The structures of colchicine, Aerosol-OT, sodium dodecyl sulfate, and structures of other two analogues are given in Scheme 1 (abbreviations are also given and A, B, and C rings of colchinoids are designated in colcemid structure). Absorption and emission spectrum was measured with Shimadzu (Model UV 1601) UV−vis spectrophotometer and Jobin Yvon-fluoromax-3. All the fluorescence emission spectra and steady-state anisotropy at a particular wavelength were corrected for the wavelength sensitivity of the detection system. In all cases, colchicine was excited at 375 nm in steady-state experiments as well as in time-resolved measurement. The details of the experimental setup for the picosecond time correlated single photon counting (TCSPC) were described in our earlier publications.[44] Far-UV CD spectra were recorded on a JASCO-810 automatic recording circular dichroism spectrophotometer. Quartz cuvettes with 1.0 cm path length were used. CD spectra were accumulated at 25 °C at a scan rate of 100 nm/min between 250 and 400 nm. Aliquots drawn at different time intervals were maintained at a final concentration of 40 μM for each solution. For size measurements, we have done dynamic light scattering measurements using Malvern NanoZS employing 4 mW He−Ne laser (λ = 632.8 nm).

3. RESULTS

3.1. Steady-State Results. Colchicine shows a normal structureless absorption maxima at 353 nm in water and also at 353 nm in methanol and ethylene glycol, whereas in dimethyl formamide λ_{abs} = 342 nm. But colchicine entrapped inside reverse micellar core exhibit absorption band at 352 nm irrespective of core solvent. Colchicine in AOT reverse micelle and in SDS reverse micelle exhibits a blueshifted excitation spectra from 363 to 355 nm with gradual addition of core solvents; the same trend is also reflected in absorption spectra with blueshift of

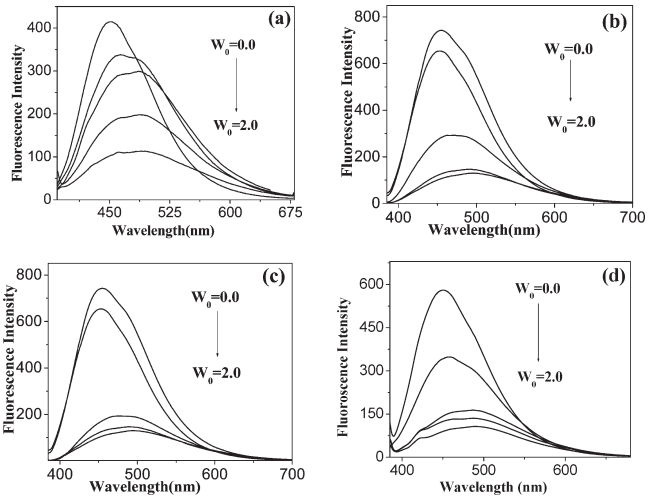

Figure 1. Plot of fluorescence emission spectra of colchicine at W_0 = 0.0, 0.5, 1.0, 1.5, and 2.0 (gradually decreasing) for (a) ethylyne glycol−AOT reverse micelle, (b) water−AOT reverse micelle, (c) methanol−AOT reverse micelle, and (d) DMF−AOT reverse micelle.

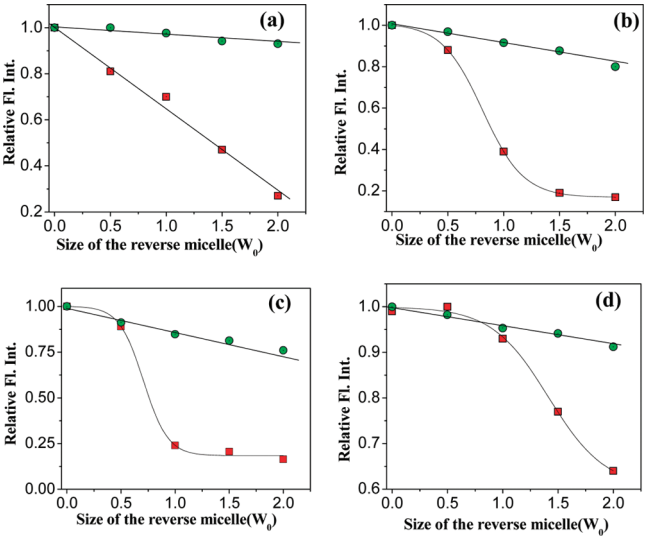

Figure 2. Plot of relative fluorescence intensity versus W_0 values at W_0 = 0.0, 0.5, 1.0, 1.5, 2.0 fot (a) ethylyne glycol−AOT reverse micelle, (b) water−AOT reverse micelle, (c) methanol−AOT reverse micelle, and (d) DMF−AOT reverse micelle (red square = colchicine, green sphere = isocolchicine).

4 nm except in the case of dimethyl formamide reverse micelle. In the case of colcemid (deacetamide colchicine) and isocolchicine (an isomer), absorption measurements give similar results compared to colchicine in all types of RMs. So from this study, we cannot differentiate between their photophysical behaviors.

Colchicine bound to tubulin shows emission maximum to a shorter wavelength at 435 nm with a large increase in intensity compared to its free analogue.[32] An identical increment is observed in our system but with a redshift at 450−480 nm regions with respect to tubulin colchicine complex (Figure 1). Colcemid does not fluoresce upon binding with tubulin and here also it does not show any fluorescence in reverse micellar solution but isocolchicine does, which is weak in nature (shown in Figure 1

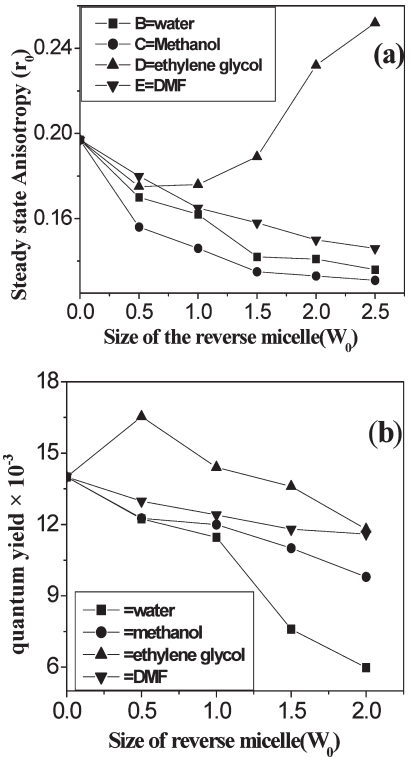

Figure 3. (a) Plot of steady-state anisotropy of colchicine versus different W_0 values. (b) Plot of quantum yield of colchicine versus different W_0 values.

in Supporting Information). After interaction with tubulin, it was reported that isocolchicine also shows fluorescence.[45] The nature of the decrement of fluorescence intensity with increase in size of the reverse micellar core is shown in Figure 2. During anisotropy measurement, we have only observed how the steady-state anisotropy value (r_0) changes with increase in W_0 value. r_0 value drops monotonically from 0.198 to ~0.13 in all reverse micelles except in ethylene glycol reverse micelle where anisotropy value gradually increases with an increase in the size of the reverse micelle (Figure 3a). But in the case of isocolchicine, a small change in r_0 value is observed (shown in Table 1 and Figure 2 of Supporting Information). We have calculated the quantum yield of colchinoids by using the gradient method.[46] We have a value of quantum yield of 0.014 of colchicine bound within the prereverse micellar core and the decreases in a regular manner with incorporation of polar core solvent inside the reverse micellar core (Figure 3b). In the case of isocolchicine, we found a lower value of quantum yield of 0.009 that is almost unaltered during the size variation of RMs core (shown in Figure 3 Supporting Information).

3.2. Circular Dichroism Study. During this study in all sets, we have kept the concentration of colchinoids accurately the same and low (4×10^{-5} M) to avoid any type of unwanted features just like dimerization of colchicine molecule[47] or any effect on ellipticity of high concentration. We have done a series of samples to check the characteristic change in the CD spectrum of colchinoids in all type of environment. By definition, molar ellipticity can be expressed as $\Delta\epsilon = \epsilon_L - \epsilon_R$ where ϵ_L and ϵ_R are molar extinction coefficients of left and right circularly polarized light and molar circular dichroism $\Delta\epsilon$ can be readily interconverted to molar ellipticity $[\theta]$ by the equation $[\theta] = 3298.2\ \Delta\epsilon$.

Table 1. Circular Dichroism Results

reverse micelle	core solvent	concentration (M)	$(\theta_{280nm}/\theta_{345nm})$ mdeg
n-heptane		8×10^{-6}	0.0
AOT with no core solvent		4×10^{-5}	2.25
AOT	water	4×10^{-5}	15.2
AOT	methanol	4×10^{-5}	8.7
AOT	ethylene glycol	4×10^{-5}	8.9
AOT	DMF	4×10^{-5}	5.2
SDS	water	5.5×10^{-5}	15.1

In water, methanol, and ethylene glycol, colchinoids exhibit two negative bands in the 270−290 nm region and in the 345−355 nm region. In these hydrogen bond donating solvents, we have studied CD spectra of colchicine in various type of reverse micelle, and one negative peak is observed rather than two distinct peaks at 280 nm and the peak at 350 nm and the later one is totally vanished. Two additional positive bands at 390−395 nm region is observed after incorporation inside AOT reverse micelle, which are absent for both the colcemid and isocolchicine. In the case of DMF, reverse micelle pattern of CD spectra is different and the ratio between the ellipticity at 280 nm and at 345 nm $(\theta_{280}/\theta_{345})$ is not so much increased as is observed in the case of other reverse micelle. Colcemid shows considerable change in CD spectra inside RM condition but isocolchicine does it differently. The CD spectrum of colcemid and isocolchicine in RM core and in various solvents are shown in Figures 5 and 6 in Supporting Information, respectively. The change in ratio $(\theta_{280}/\theta_{345})$ for isocolchicine is very small, but for colcemid the change is good just like colchicine (shown in Tables 3 and 4 in Supporting Information). In the case of SDS reverse micelle, although the ratio is increased, the two additional peaks at the longer wavelength region are absent. All the values of the ratio at different W_0 values are given in Table 1 and the CD spectrum of colchicine are shown in Figure 5a−c.

3.3. Time-Resolved Measurements. We have tried to check the lifetime of all the colchinoids in various solvent even in viscous solventlike ethylene glycol but we were unable to make it. We have only one important data about the lifetime of colchicine molecule, which is the lifetime of tubulin−colchicine complex. This complex has a lifetime of 1.14 ns at an ionic strength of 0.1 M and 1.2 ns at 1.0 M.[9] As we expected, we could measure the lifetime of the entrapped colchicine molecule in various types of reverse micelles. In the case of hydrogen bond-donating solvent as well as in non-hydrogen bond-donating solvent, we have observed a similar type of trend in decay pattern except in the case of high viscous glycol reverse micelle. We have measured the lifetime of isocolchicine, which was almost unchanged as W_0 increases for all types of RM (shown in Table 2 and in Figure 4 in Supporting Information) but in the case of colchicine at $W_0 = 0.0$, lifetime is 282 ps and decreased as the W_0 value is gradually increased from 0.0 to 2.0 in a regular manner. In the case of glycol reverse micelle, there is a sudden increase at $W_0 = 0.5$, but after that it also follows the normal decreasing trend with an increase in size of the reverse micelle. Here in our study we have found a huge increment in lifetime of colchicine embedded inside a reverse micelle made of a single chain surfactant sodium dodecyl sulfate and 1-hexanol and water. The lifetime of colchicine in SDS reverse micelle at $W_0 = 9.9$ is 2.45 ns and at $W_0 = 15.1$

Table 2. Time-Resolved Results of Colchicine Inside Various Reverse Micelles[a]

reverse micelle	<τ> (ps) W_0 = 0.0	<τ> (ps) W_0 = 0.5	<τ> (ps) W_0 = 1.0	<τ> (ps) W_0 = 1.5	<τ> (ps) W_0 = 2.0
only n-heptane					
AOT−water	282	207	170	154	134
AOT−MeOH	282	198	159	141	102
AOT−DMF	282	225	186	165	130
AOT−ethylene glycol	282	409	238	179	162

reverse micelle	<τ> (ps), W_0 = 9.9	<τ> (ps), W_0 = 15.1
SDS−water	2450	2510

[a] <τ> (ps) is average lifetime in picoseconds.

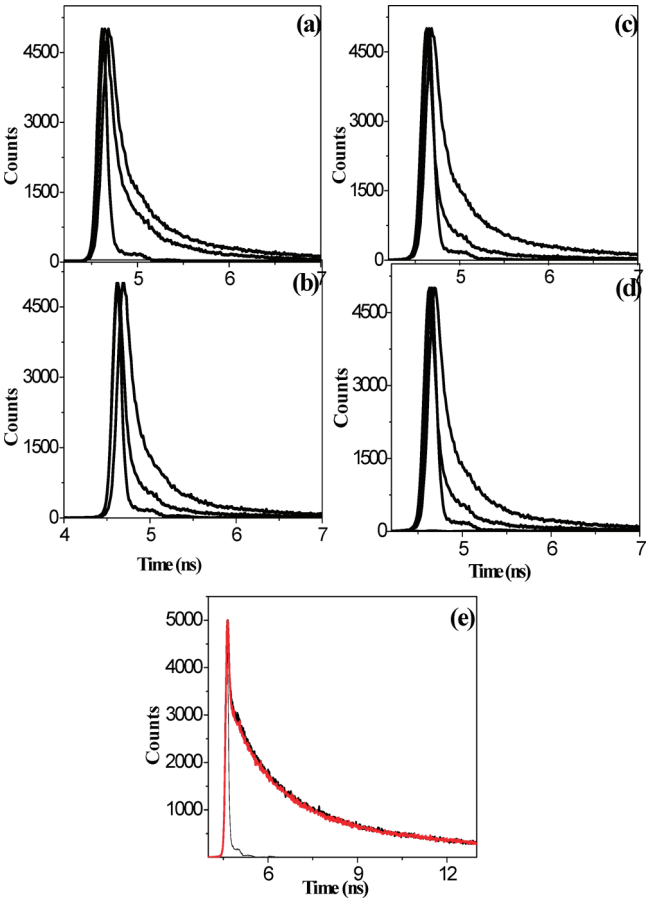

Figure 4. Lifetime quenching plot of colchicine at W_0 = 1.0 and 2.0 for all type of reverese micelle of AOT. (a) Water−AOT reverse micelle; (b) ethylyne glycol−AOT reverse micelle; (c) methanol−AOT reverse micelle; (d) iimethyl formamide−AOT reverse micelle. (e) SDS reverse micelle at W_0 = 9.9 (red) and at W_0 = 15.1 (black).

is 2.51 ns, which are almost constant. The fluorescence lifetimes were taken at emission maxima for colchicine and isocolchicine at various reverse micellar condition using different core solvent. The decays of both these compounds were found to be biexponential and are shown in Figure 4a−d and in Supporting Information. The lifetime decay parameters for colchicine are shown in Table 2. Average lifetime was taken by the following equation where τ_1

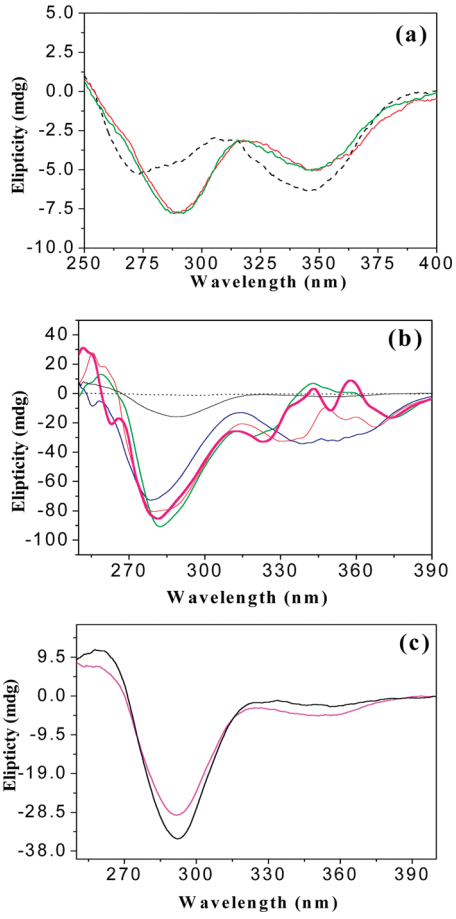

Figure 5. (a) Circular Dichroism spectra of colchicine in water (black dashed) in methanol (green line) and in ethylene glycol (red line). (b) Circular dichroism spectra of colchicine in water reverse micelle (green line), in methanol reverse micelle (bold pink line), in ethylene glycol reverse micelle (red line), in DMF reverse micelle (black line), and at W_0 = 0.0 (blue line). (c) Circular dichroism spectra of colchicine in SDS reverse micelle at W_0 = 9.9 (black line) and W_0 = 15.1 (pink line).

and τ_2 are two components of lifetime decay and a_1 and a_2 are the corresponding weightage to the average decay

$$\langle\tau\rangle = \tau_1 a_1 + \tau_2 a_2$$

3.4. Size Measurement with Temperature. In the case of dilute solution, the local fluctuations in the dielectric constant value of the medium over an entire scattering volume V resulted from scattering of light out of incident beam, and the fluctuations reflect directly the size of the particle suspended in the solution. We have done DLS measurement of all type of reverse micelle at W_0 = 0.5 to 2.0 for AOT type reverse micelle and in the case of SDS reverse micelle we have taken data from literature.[48] Water and methanol reverse micellar size at maximum water content are 4.0 and 3.0 nm, respectively. In the case of glycol, reverse micelle size of the reverse micelle vary from 1.3 to 9.0 nm at W_0 = 0.5 to 2.0. SDS reverse micellar size at W_0 = 9.9 and W_0 =15.1 remain very close to each other at 14.1 and 15.1 nm, respectively. The table of number average size distribution of reverse micelle at particular W_0 values is given in Table 4.

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4. DISCUSSION

4.1. Absorption Studies. Being a nonfluoroscent compound in bulk solvent, colchinoids creates too many problems to make any type of comparison regarding the polarity of the environment in which it is located inside a nanocavity. We can consider only the UV-absorption spectra of all colchinoids in various solvent to study their photophysical behavior. It shows a normal structureless absorption maxima at 353 nm in water for colchicine and it was reported that the normal near-UV absorption band of colchicine is actually composed of two $\pi-\pi^*$ transitions, one at 360−390 nm range and the other at 350−325 nm range.[49] The near-UV band of colchicine in solvents of different dielectric constant was studied and was reported that it show a hypsochromic shift about 12 to 13 nm varying dielectric constant from water, methanol, to dioxan and so forth.[33] In Dimethyl formamide, λ_{abs} of colchicine at 342 nm is observed, but in water, methanol, and ethylene glycol λ_{abs} of colchicine is around 352 nm. The other two colchinoids show similar trends in their absorption behavior. Isocolchicine shows 8 nm hypsochromic shifts while colcemid shows 12−13 nm hypsochromic shift for changing the solvent dielectric constant from water to dimethyl formamide. Now in AOT−RM irrespective of any core solvent, λ_{abs} of colchicine and colcemid are observed at 352 nm. But in the case of isocolchicine, it was observed at 349 nm. In the case of DMF reverse micelle, the shift of UV band is 11 nm for colchicine and colcemid and 8 nm for isocolchicine when these are trapped inside the reverse micelle but in other reverse micelles this shift is almost absent for colchicine and colcemid. So from this UV data we can make a proposal that the colchinoids are located in a similar type of environment inside the various type of reverse micelle in which the micropolarity as well as the microviscosity sensed by them is same irrespective of the core solvent. This is only possible if colchinoid molecules make some specific type of interaction with the headgroup of the surfactant molecule. One important observation in absorption spectra is that there is a blue shift of λ_{abs} of 4 nm of colchicine trapped in reverse micelle after gradual addition of core solvent. Previously, Bane et al. have reported that in bulk hydrogen bond-donating solvent the λ_{abs} value of colchicine is blueshifted compared to nonhydrogen bonding solvent, inferring that there is a H-bond formation between the carbonyl carbon and the solvent.[20] This occurs due to the $\pi-\pi^*$ charge transfer transition from carbonyl moiety to the tropone ring being less facilitated in the presence of H-bonding.

4.2. Circular Dichroism Study. The optical activity shown by (7S)-colchicine and (7R)-colchicine appear to originate from the phenyl-tropolonic system (A and C rings) rather than the C-7 chiral center of ring B.[40,50] No CD absorption was observed for AOT and SDS surfactant molecule having no chirality. CD spectra in water and in all reverse micellar systems of natural (7S)-colchicine show wavelength range between 250 and 400 nm. The CD spectrum of natural (7S)-colchicine in water gives a major negative band with a maximum at about 345 nm and a minor negative band at about 272 nm. The band at 345 is for the methoxytropone ring and the peak at 275 nm is assigned for the K-band of the biaryl ring. The CD spectra of colchicine in an AOT−*n*-heptane mixture exhibit a considerable change in the nature of the spectrum, giving a red-shifted higher energy band at 280 nm with larger intensity than the low-energy band centered at 343 nm. Now after addition of water, the low-energy band just vanished, giving two positive peaks at the same wavelength region. The same observation happened in all types of reverse micelles. Isocolchicine having a different structure shows an abnormal CD spctra in which the change in intensity at higher and lower energy band is much less when kept in free solvent as well as in reverse micellar solution. On the other hand, colcemid shows similar sharp change in CD spectra as done by colchicine, implying the same type of interaction between surfactant molecule and them. In SDS reverse micelle, only the positive peaks are absent. In the case of tubulin−colchicine complex, this negative band either vanished or was greatly reduced.[27] Bane et al. had reported that such a dramatic change in CD spectra of colchicine when bound to the protein is possible only if there is a considerable conformational change occuring itself in the colchicine molecule.[50] Also, this conjecture was further supported by several aspects of colchicines−tubulin interaction, for example, the near planarity of the second conformer can now produce an extended resonance conjugation, leading to enhanced fluorescence.[32] The near planarity decreases chirality (helicity) of the ring system leading to suspension of the rotational strength of colchicine at 340 nm. In our system, we have observed more or less the exact incident for colchicine and colcemid that we have mentioned above. So here also we can make a strong conclusion that when these two compounds are entrapped in an AOT−*n*-heptane mixture or in an AOT reverse micller core or any other reverse micellar core, it certainly undergoes a conformational change to its more planar conformer from its nonfluorescent boatlike conformer. In previous studies,[50,51] it was already reported that the tubulin binds to the tropolone moiety of these compounds, which reduces the intensity of the band at 340 nm or totally vanishes this negative band. In our case, colchicine and colcemid in water give two distinct negative bands in which the low-energy band at 345 nm is reduced or totally vanished inside prereversemicellar state and reverse micelle. Again the intensity ratio $(\theta_{280}/\theta_{345})$ between the band at 272−280 and the band at 345 is drastically increased in reverse micellar or prereversemicellar condition with respect to free analogue in water. This happened only when there was a hydrogen-bonding environment.[41] From the CD spectra of colchicine and colcemid in water, methanol, and ethylene glycol solvent, we can see that the ratio $(\theta_{280}/\theta_{345})$ were almost 1 or near about 2 and in reverse micellar condition of that core solvent the value of the ratio $(\theta_{280}/\theta_{345})$ increased to 5−12 range. This increment clearly indicates that inside reverse micellar core there is strong hydrogen bonding that is between the hydrogen of the acyl chain and the oxygen of the sulfate group of the surfactant along with the hydrogen bonding between the carbonyl oxygen and the hydrogen from the solvent. But in the case of nonprotic solvent DMF, there is no significant increase in intensity ratio of the two bands at 272 and 345 nm. So in hydrogen bond donating solvent, the colchicine molecule is located in the core or near the core of reverse micelle making a hydrogen bond network with solvent.

Now the small change for isocolchicine indicates that there should not be considerable conformational change during its entrapment inside RMs. It was previously reported that the interconversion of tropone C ring of isocolchicine does not give any conformer in which the phenyl ring (A) and the troponoid ring (C) are nearly plannar.[45] It results in two conformers in which both are in "skewed boat form" and the biaryl angle between A and C ring are 45 and 60°. Finally, we can demonstrate that although there may be a strong interaction between the head group of AOT molecule and isocolchicine upon entrapment, there should not be any

Table 3. Steady State Anisotropy (r_0) at Different Reverse Micellar Condition

reverse micelle	r_0 (W_0=0.0)	r_0 (W_0=0.5)	r_0 (W_0=1.0)	r_0 (W_0=1.5)	r_0 (W_0=2.0)
AOT−water	0.197	0.162	0.142	0.141	0.136
AOT−MeOH	0.197	0.146	0.135	0.133	0.131
AOT−DMF	0.197	0.165	0.157	0.149	0.145
AOT−glycol	0.197	0.180	0.189	0.232	0.252

conformational change of this analogue as shown in the case of the other two analogues.

4.3. Fluorescence Study. Now the dramatic increase in fluorescence intensity of colchicine after confinement in pre-reverse micellar condition and also in reverse micellar condition can be attributed to an increase in microviscosity as well as a decrease in micropolarity around the colchicine molecule. Colcemid does not show any fluorescence in free solvent as well as inside RMs core but isocolchcine shows a weak fluorescence upon entrapment inside RMs core. It was reported that in the case of colchicine molecule, two conditions are very essential as well as necessary. These are the threshold viscosity and hydrophobicity of the environment to fluoresce, providing there should be some sort of immobilization of the drug molecule. The viscosity can be achieved in two ways, (1) by adding viscous solvent or (2) by providing constraints by specific or covalent interaction with some protein, like tubulin. An appreciable amount of (about 20−30 nm) redshift in fluorescence spectra is observed between W_0 = 0 and W_0 = 2.0 condition for all the reverse micelle irrespective of nature of core solvent. From this result here, we can again make an inference that the colchicine molecule is located in such a location that is affected by the addition of core solvent. Colchicine is insoluble in *n*-heptane and highly soluble in all the polar solvent water, methanol, ethylene glycol, and dimethyl formamide. So now it is very clear that colchicine molecule is entrapped inside the reverse micellar core or in the headgroup−water interface region facing the inner pool. The same conclusion can be drawn for the isocolchicine molecule regarding the location of this molecule. But the decrement in intensity is much less, which can again be explained by two nonplanar conformations mentioned before, that is, after or before confinement there will be a little gain in planarity (a little gain of resonance conjugation) and there also be a little loss of planarity (a little loss of resonance conjugation) with increase in size of RM core from W_0 = 0.0 to 2.0.

A different fact now has to be introduced for demonstrating the nonfluorescent nature of colcemid after and before confinement, while only the acetyl group is absent compare to colchicine. From CD spectra, we have seen that the change in conformation is almost like colchicine. In the case of colchinoid−tubulin binding, it was proven that the substituent at B-ring has a profound effect on the fluorescence properties of these drug molecules and at the same time it is also true that the free biaryl analogue (AC) can also show some sort of fluorescence after binding with tubulin.[33] Now one question should arise in mind: only the extended resonance conjugation between the trimethoxy phenyl ring and troponoid ring is not responsible for enhancement of fluorescence of colchicine. If this was the case, then Colcemid should show fluorescence after entrapment inside RM core because it has already shown similar type of conformational change like colchicine. Now simply one can

Table 4. Size Measurement of Different Reverse Micelle at Different W_0 values

reverse micellar (AOT) core solvent	size (nm) W_0 = 0.5	size (nm) W_0 = 2.0
water	0.7	4.0
methanol	1.0	3.0
ethylene glycol	1.3	9.0
DMF	1.2	2.6

reverse micellar (SDS) core solvent	size (nm) at W_0 = 9.8	size (nm) at W_0 = 15.1
water	11.8	10.4

consider that the N-acetyl group also has a parallel effect on their fluorescence properties. This supposition is somehow rechecked by the generation of fluorescence of isocolchcine which has the N-acetyl group (same as in colchicine) as well as the two rings A and C.

But under this restricted environment, colchicine molecule has gained the more planar conformation that provides extended resonance conjugation to enhance the fluorescence intensity as seen from CD study. Now the decrement in fluorescence intensity after gradual addition of polar core solvent is due to many factors. One is the polarity of the medium increases, another is the microviscosity of the reverse micellar core decreases, and the last one is the constraint of the local environment of colchicine decreases, which helps the colchicine molecule to get its boatlike conformer. In hydrophobic environment, fluorescence property of colchicine increases[32] but here the little amount of hydrophobicity delivered by the surfactant decreases after addition of polar core solvent. We have calculated quantum yield of colchicine, steady-state anisotropy and fluorescence intensity with increasing W_0 value and all these parameters are decreasing in all cases in a regular manner except in the case of ethylene glycol reverse micelle. In these cases, two opposing factors are operating to control the fluorescence property of colchicine. After addition of highly viscous ethylene glycol, size of the reverse micelle increases and at the same time the microviscosity of the reverse micellar core also increases. As increased internal volume of reverse micellar core gives more space to colchicine to be puckered to its boatlike conformer, at the same time microviscoity of the microregion certainly increases which may hinder this puckering of the conformer and decrease the rate of internal conversion reaction.[32] Then a situation meets where the increasing factor become dominant over the decreasing factor at W_0 = 0.5 of ethylene glycol reverse micelle. After gradual addition of core solvent, the decreasing factor becomes dominant over the increasing one. In Table 3, we can see the steady-state anisotropy value of colchicine entrapped in various type of reverse micelle. But the picture is different for isocolchicine, which has lower quantum yield than colchicine, and the value is almost unaltered during the size change of reverse micelle. In AOT−*n*-heptane mixture, the value of r_0 is highest, 0.196, and then gradually decreases to 0.13 as expected from the location of the colchicine molecule in all reverse micelle except in ethylene glycol reverse micelle where the value of r_0 gradually increases after gradual addition of this solvent. For isocolchicine, the value of r_0 also vary during addition of core solvent. In the case of colchicine, decrement in fluorescence intensity occurs in a sigmoidal nature in all cases again except in ethylene glycol

reverse micelle, but for isocolchcine it is linear irrespective of nature of reverse micelle and the rate of decrement is very low. In ethylene glycol, reverse micelle of the decrement for colchicine occurs in a straight line and in water reverse micelle, the rate of fall is highest because the size of the reverse micelle increased from $W_0 = 0.5$ (1 nm) to $W_0 = 2.0$ (4.0 nm) as well as the polarity of water is very high, thus both the effects caused sharp reduction in fluorescence. In the case of methanol reverse micelle, the polarity is less than in water and viscosity is lower than water and the overall effect is same as in water reverse micelle. In DMF reverse micelle, more or less the same thing is observed with a little difference in the structure of the emission spectra. This change in emission spectral pattern in such nonhydrogen bonding solvent may be due to a different distribution coefficient of colchicine molecule in between the polar headgroup region and the inner polar pool. Ethylene glycol reverse micellar size varies from 1.3 to 9.0 nm when it goes from $W_0 = 0.5$ to $W_0 = 2.0$. Here the size factor is greatly enhanced, which is actually counterbalanced by the increasing viscosity effect in this particular reverse micelle. If we give some inner look on this result of ethylene glycol reverse micelle for colchicine, then we can see that although the viscosity is very high (r_0 value is 0.25 at $W_0 = 2.0$ of glycol reverse micelle) in a nanocavity region, the fluorescence intensity is very less compared to that of in prereversemicellar condition or at $W_0 = 0.5$.

By using reverse micelles except glycol reverse micelle, we cannot set the trick but with the help of the glycol reverse micelle we can say that the conformational change is a main factor responsible for the dramatic increase in fluorescence intensity. To give support for this statement, we can see that the microviscosity values inside water reverse micelle at $W_0 = 2.0$ are in a range of 150 to 190 cP with a polarity comparable to chloroform, dichloromethane, or pyridine solution where the threshold viscosity for fluorescence of colchicine is only 3 cP.[32,49b] Under such a high viscous condition, it should give considerably high-fluorescence intensity but it cannot. Because increase in size of the reverse micelle, colchicine loses its near planar conformation irrespective of the viscosity of the solvent surroundings the colchicine. If there is covalent bonding between the surfactant and colchicine, the same type of increment should be observed in SDS micelle and also in CTAB micelle. There is also no fluorescence property of colchicines observed in micellar condition. But again we have used SDS reverse micelle and had an abnormal increase in fluorescence intensity even greater than that in all type of AOT reverse micelle. By all this cross checking one can infer undoubtedly that under constraint condition colchicine become more of a planar conformation and begin to fluoresce.

Now, if we consider all the discussion on colchicine along with its two different analogues we have obtained a different story regarding their photophysical behavior. Colcemid does not fluoresce after entrapment inside RM core, while isocolchicine shows weak fluorescence after nanocavity embedment. The reason behind weak nonaltering fluorescence behavior for isoanalogue and no fluorescence for colcemid are hidden in their structural feature along with constraint effect given by nanocavity of RM core. These results undoubtedly suggest for us to conclude that there must be a different mode existing for three colchinoids inside the nanocavity.

4.4. Time-Resolved Study. All our results were verified by time-resolved studies of colchicine in various reverse micelles. To the best of our knowledge, there is no data of fluorescence lifetime in any type of solvent even in glycerol. The lifetime of colchicine after binding with tubulin is 1.14 ns.[34] In our system, it lies between much lower regimes of 282 ps for colchicine and 262 ps for isocolchicine. Also, there is gradual decrease in lifetime for the former one while the other is almost constant with gradual increase in size of the reverse micelle. For colchicine in ethylene glycol reverse micelle, there is increment at $W_0 = 0.5$ and then again decreases. The reason behind this fact was already discussed earlier. This TCSPC data is nothing but a proof of our earlier proposition. However, one thing is observed that is very interesting as well as difficult to explain. That is colchicine gives a higher value of lifetime after incorporated inside SDS reverse micelle, which is about 2.51 ns. We think that is due to colchicine molecule entrapped more tightly in this single chain surfactant and it somehow manages to place itself to more restricted microenvironment where both the increment effect, constraint, and viscosity play together upon molecular skeleton of colchicine because the value of steady state anisotropy is considerably high 0.216 in SDS reverse micellar system. But in the case of isoanalogue, we got 296 ps in SDS reverse micelle and this little enhancement is attributed due to higher viscosity of SDS reverse micellar solution.

Finally we have arranged our considerations to prove our results about the increment of fluorescence intensity as well as lifetime of colchinoids in a single statement that it is due to the right puckering of the structure of colchinoids achieving more planar conformation, which is given by nanocavity of reverse micelle as well as the presence of B-ring substituents that also have a pronounced effect to enhance the fluorescence properties.

5. CONCLUSION

In our present work, we are going through various problems regarding absence of fluorescence property of the drug molecule in normal bulk solvent. So our main attempt was to manifest its fluorescence and compare it under different conditions. Under nanocavity effect, various properties of colchinoids like anisotropy, conformation, lifetime, and so forth were investigated. We found results similar to tubulin−colchinoid interaction when the colchinoids were incorporated into reverse micelle. We did not give any comparison between the protein−colchinoid interactions with the colchinoid-revese micelle interaction. But with the help of useful results regarding various structural and photophysical properties of colchinoids in tubulin−colchinoid interaction, we gave a simple look behind the unusual behavior of colchicine and its two analogues when entrapped inside RM core. In conclusion, it was demonstrated that due to right puckering of the structure of colchinoids achieving more planar conformation, which was given by constraint of nanocavity as well as the presence of B-ring substituent, which also have a pronounced effect to enhance their fluorescence properties.

■ ASSOCIATED CONTENT

Supporting Information. Plot of variation of steady-state anisotropy and quantum yield with W_0 of isocolchicine in various RMs and CD spectrum of colcemid and isocolchine in various RMs, This material is available free of charge via the Internet at http://pubs.acs.org.

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