^{1}H-NMR and FT-IR study of the state of melatonin confined in membrane models: location and interactions of melatonin in water free lecithin and AOT reversed micelles

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Abstract
The state of melatonin confined either in dry lecithin or bis(2-ethylhexyl) sulfosuccinate sodium salt (AOT) reversed micelles has been investigated by ^{1}H-NMR and FT-IR spectroscopies as a function of the melatonin to surfactant molar ratio (R). The analysis of experimental results leads to hypothesize that, independently of R and the surfactant nature and as a consequence of anisotropic melatonin/surfactant interactions, melatonin is totally solubilized in reversed micelles and mainly located by opportune orientation in the nanodomain constituted by the surfactant head groups. The absence of significant spectral changes related to the protons linked to the first carbon atoms of surfactant alkyl chain, indicates a scarce insertion of melatonin into the so-called micellar palisade layer. The possible biological implications of the peculiar solubilization state of melatonin in reversed micelles are discussed.

Keywords: Melatonin, membrane models, reversed micelles, confinement effects

Introduction

Melatonin (MLT), *N*-acetyl-5-methoxytryptamine, is a hormone having an indolic structure[1] which is principally secreted during the night from the mammal pineal gland (epiphysis). It mediates various neuroendocrine and physiologic cellular processes and controls some neural circadian effectors regulating sleep[2] and seasonal reproduction cycles[3] as well as other endocrine glands, and has anti-tumoral and anti-degradation properties. Moreover, MLT is the unique hormone which possesses strong anti-oxidant properties. In particular, it acts as a powerful inhibitor of free radicals and of oxygenated active species resulting from the exposure of living

organisms to external agents such as ultraviolet radiations, ozone, tobacco, alcohol, asbestos and pesticides.

Oxygenated active species are also generated from the breathed oxygen. Up to 5% of the oxygen absorbed from mitochondrions comes out as oxygenated free radicals. At muscular level they damage and destroy membranes, disarm enzymes, and alter the genetic map; all these effects occur in a very short time (about 1 ns). In absence of anti-oxidant agents, their action is devastating and relentless[4] determining irreversible chemical changes and could lead to a series of diseases such as cancer,[5,6] AIDS,[7] cataract[8] and cardiac,[9] Parkinson's and Alzheimer's[10,11] diseases. MLT is resulted five times more effective than glutathione in capturing hydroxyl radicals and five hundreds times more effective than dimethyl sulfoxide in safeguarding chromosomes from radiation induced damages.[12] Besides, it inactivates lipoperoxyl radicals[13] and nitrogen monoxide.[14,15]

From a molecular point of view, MLT is a small size amphiphilic substance. For this reason, it is soluble both in fats[16] and in water,[17] preferentially located at hydrophilic/hydrophobic interfaces and can easily cross all the anatomic barriers including hematoencephalic and placental ones. These properties make melatonin able to protect all the cellular structures from oxidant agents.

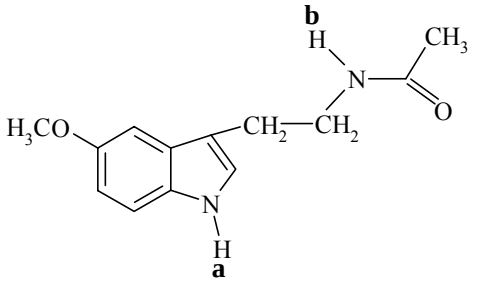
Obviously, a more deep comprehension of the MLT properties in living systems requires a detailed knowledge of its interactions with cellular membranes. This because they are the driving force of its preferential localization and diffusive properties. This consideration is at the basis of previous studies on MLT with simple model systems which could simulate possible biological localization sites. Some investigation on the localization of MLT in systems characterized by the presence of aqueous, apolar and polar micro domains, as inversed micelles including water in the micellar core,[18] phospholipidic layers[19] and cyclodextrins[20] in aqueous medium have been reported. The results indicate that MLT locates in the interface between the polar head region and the water enclosed in the micellar core,[18] between the two layers in phospholipide bi-layers[19] and it is inserted into the hydrophobic cavities of cyclodextrins.[20]

On the other hand, nervous cells of the brain cannot be regenerated and those hit from free radicals are irremediably destroyed. As years go by and some neurons are lost, mental functions are compromised with consequent degenerative damages. Prevention of these pathologies is the assumption of anti-oxidant agents.[21] However, the neurons synaptic portion is a quite anhydrous environment separated from aqueous compartments through hematoencephalic barrier. This prevents the entrance of harmful polar substances but, unfortunately also of most anti-oxidant agents. In order to investigate the MLT behavior in such environments, it seems more appropriate to choose totally anhydrous reversed micelles as water-free membrane models. In this work, we investigate the interactions and the location of MLT in L-α-phosphatidylcholine (lecithin) or bis(2-ethylhexyl) sulfosuccinate sodium salt (AOT) reversed micelles dispersed in carbon tetrachloride as a function of the MLT to surfactant molar ratio (R). A schematic representation of these molecules is shown in Figure 1.

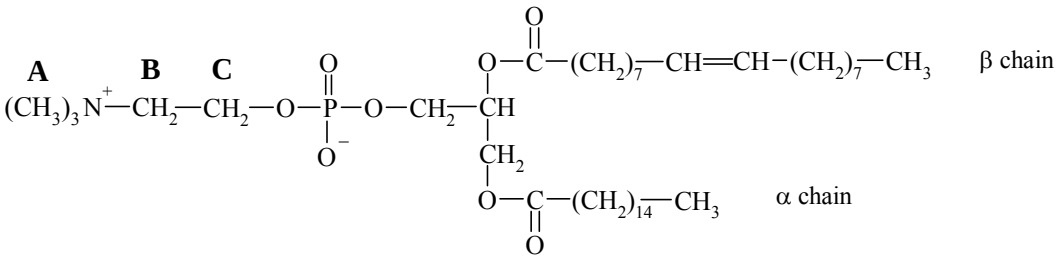
Reversed micelles are one of the most interesting structure of nanometric dimensions (1-20 nm)[22] formed by some surfactants when dissolved in apolar solvents at concentrations higher than a threshold value called critical micellar concentration (cmc). They can be schematically represented as globular aggregates constituted by an internal core made up by surfactant

hydrophilic heads and an external region formed by the surfactant alkyl chains. It is worth to note that reversed micelles share many fundamental properties of bio-membranes 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.

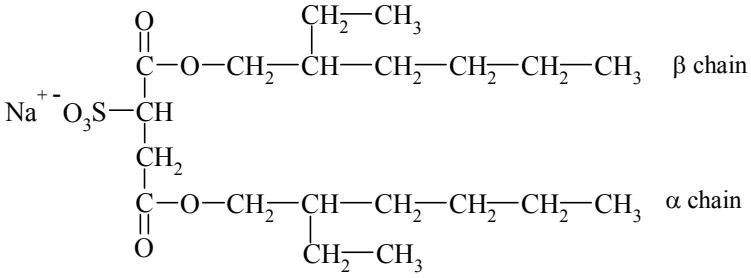
^{1}H-NMR and IR spectroscopies were chosen for this investigation because they have been proved to be suitable techniques for investigating the state of solute molecules entrapped in reversed micelles.[23]



MELATONIN



LECITHIN



BIS (2-ETHYLHEXYL) SULFOSUCCINATE SODIUM SALT

Figure 1. Molecular structures of MLT, LCT and AOT.

Results and Discussion

In our study, solutions of reversed micelles were formed by dissolving Soybean lecithin (LCT) or bis(2-ethylhexyl) sulfosuccinate sodium salt (AOT) in carbon tetrachloride at fixed surfactant

concentration (0.1 mol/kg). Appropriate amounts of MLT were added to these solutions to obtain samples at various values of the molar ratio R (R = [MLT]/[surfactant]).

MLT solubility, expressed as the maximum R value, is 0.31 in LCT/CCl_4 and 0.18 in AOT/CCl_4 solutions. It is worth to note that the MLT solubility in LCT/CCl_4 solutions is larger than that in AOT/CCl_4 ones suggesting a higher affinity of MLT for LCT reversed micelles.

^{1}H-NMR investigation of MLT/lecithin/CCl_4 system

The ^{1}H-NMR spectra of LCT/CCl_4, MLT/CCl_4 and MLT/LCT/CCl_4 systems allow the attribution of the peaks of the lecithin and melatonin protons (see Figure 2). Detectable changes of the chemical shifts (c.s.) of some MLT and lecithin (LCT) protons occur in the micellar system. In particular, measurable c.s. variation concerns the signals of the protons A-C of LCT and of the indolic and amidic protons of MLT (a and b, respectively).

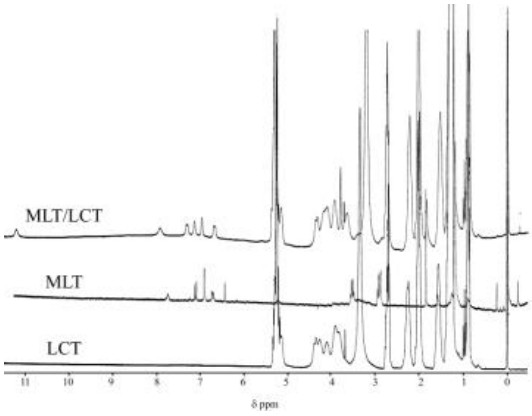


Figure 2. Comparison between the ^{1}H-NMR (CCl_4) spectra of MLT, MLT/LCT (R=0.31) and that of LCT systems.

A perusal of all the ^{1}H-NMR spectra and of the proton chemical shifts (ppm from TMS) reported in Table 1, clearly reveals the establishment of an interaction between MLT and lecithin. In fact, a dramatic downfield shift is observed for the indole (a) and the amidic (b) NH protons of MLT with respect to those observed in CCl_4 solution (7.74 and 6.45 ppm, respectively).

Table 1. Chemical shifts ppm from TMS) of lecithin A, B, C proton peaks and MLT indolic (a) and amidic NH (b) protons at various R

R	A (LCT)	B (LCT)	C (LCT)	a (MLT)	b (MLT)
0	3.34	4.26	3.81		
0.10	3.26	4.21	3.72	11.39	8.11
0.17	3.24	4.16	3.67	11.27	7.97
0.22	3.21	4.12	3.63	11.21	7.91
0.31	3.17	4.06	3.55	11.16	7.86

On the other hand, they are very close to that observed in the spectrum recorded using exadeuterodimethylsulfoxide (DMSO) as solvent (10.67 and 7.97 ppm, respectively).

This finding suggests that these protons lie in a polar nanodomain. Moreover, some changes of the position (downfield shift) and widths (broadening) of the aromatic proton peaks accompanying the confinement of the indolic moiety of MLT in LCT micelles are also observed. As counter part, the main c.s. variations of LCT protons regard those of the polar head. This is emphasized by the upfield shift of LCT A, B, C protons of the surfactant polar head by increasing R. Plotting the chemical shifts of lecithin A, B, C protons as function of R, a good linear correlation is found. In particular, it can be noted that the small but detectable upfield shift with R is more pronounced for the C protons (see Figure 3).

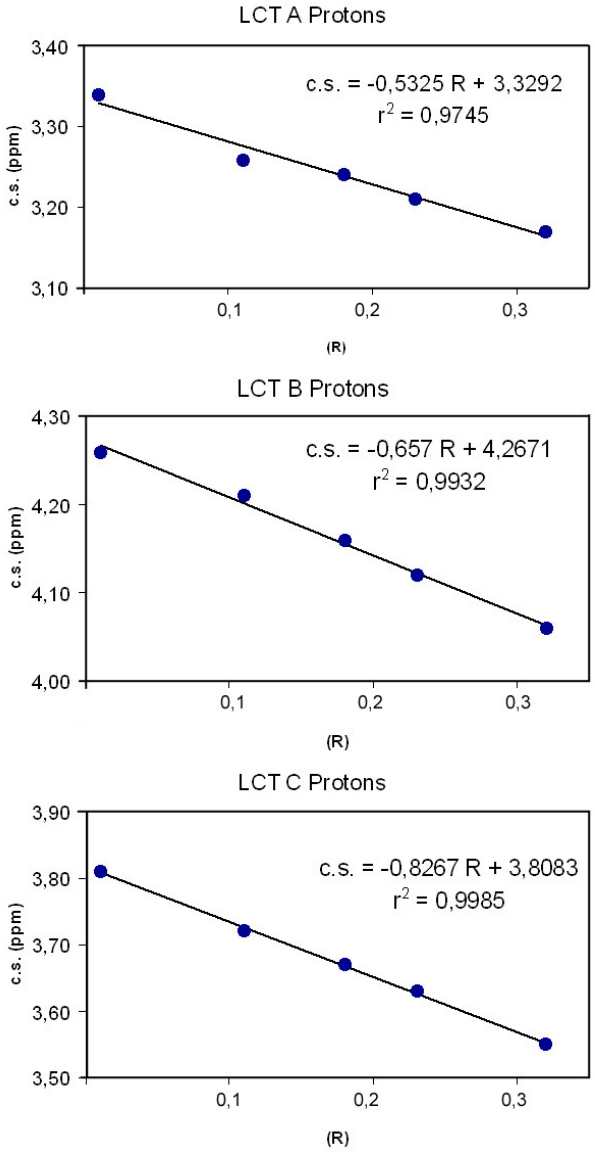


Figure 3. Chemical shift (c.s.) of LCT A, B, C protons as function of R.

This monotonous behavior indicates that, in all the investigated R range, the addition of melatonin progressively influences the core of lecithin reversed micelles, i.e. an internal core constituted by melatonin surrounded by lecithin molecules is never formed.

Similarly, the linear downfield variations of the c.s. of the indolic (a) and amidic (b) NH protons with 1/R, shown in Figure 4, indicates that MLT progressively interacts with lecithin head group mainly through its indolic and amidic NH groups. This also means that melatonin molecules are entrapped in the palisade layer of lecithin reversed micelles so that these groups are nearby the hydrophilic micellar core.

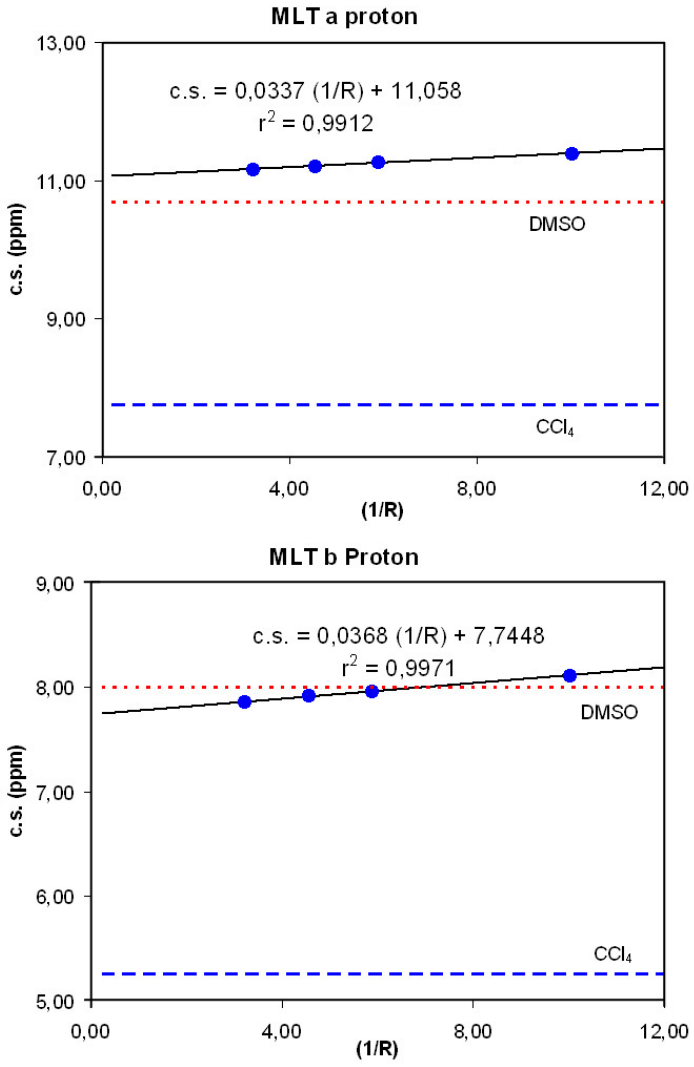


Figure 4. Chemical shift of MLT a and b protons as function of 1/R. Dashed blue and dotted red lines represent the c.s. of the MLT NH protons in DMSO and in CCl_4, respectively.

An inspection of Figure 4 suggests that the indolic NH group is engaged in a hydrogen bond stronger than that in DMSO while the amidic NH group is involved in hydrogen bond of

comparable strength. The small linear downfield shift of the NH protons with 1/R indicates minor variation of the MLT/LCT hydrogen bond interaction with this parameter.

FT-IR investigation of MLT/ LCT/CCl_4 system

Further information on the interactions between MLT and lecithin were achieved by an FT-IR investigation.

By comparing the NH stretching band (3000-3600 cm^{-1}) of MLT in CCl_4 solution with that of pure MLT and MLT/LCT/CCl_4 system at various R values (Figure 5), it can be observed that the two quite sharp bands at 3460 and 3490 cm^{-1} due to MLT indolic and amidic NH stretching of monomeric MLT, respectively, change in broad bands shifted to lower wavenumbers. These shifts are consistent with the existence in both pure MLT and MLT confined in reversed micelles of strong MLT-MLT or MLT-LCT H-bonds involving indolic and amidic NH groups.

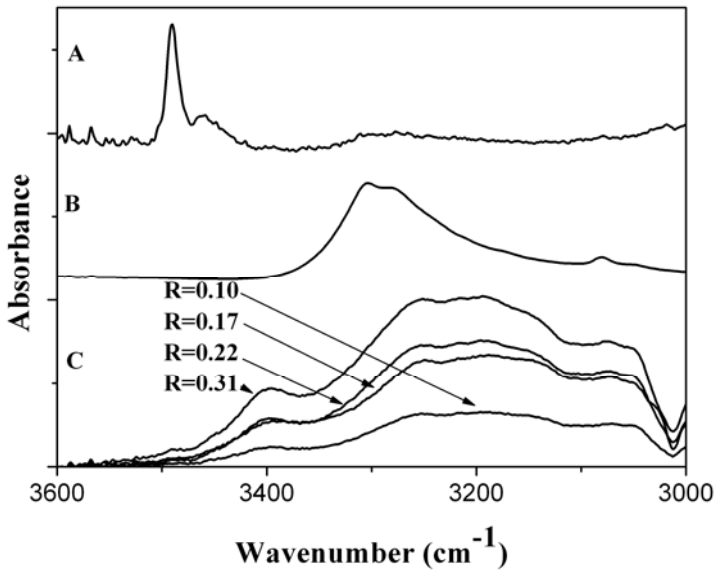


Figure 5. Comparison between IR spectra of MLT dispersed in CCl_4 (A), pure MLT (B) and MLT/lecithin/CCl_4 system at various R values (C).

It must be also noted that the NH band of MLT confined in LCT reversed micelles is broader than that of pure MLT. This finding suggests the presence in the MLT/LCT/CCl_4 system of a spectrum of H-bonded MLT populations wider than that in pure MLT.

As already shown by the NMR investigation, the occurrence of MLT-LCT H-bonds is also shown by the PO_4^- antisymmetric stretching bands (1262 cm^{-1}) of lecithin; it in fact shifts towards lower wavenumbers while increasing R values (Figure 6).

The monotonous variation of the frequency of the PO_4^- band maximum (F^*) with R suggests that the addition of melatonin involves a parallel increase of the fraction of surfactant PO_4^- groups interacting with melatonin.

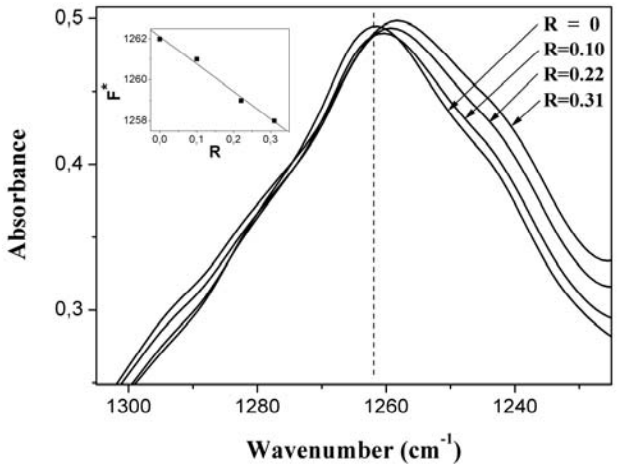


Figure 6. PO_4^- antisymmetric stretching band of MLT/lecithin/CCl_4 system at various R values. In the inset is reported the frequency (F*) at the band maximum as a function of R.

^{1}H-NMR investigation of MLT/AOT/CCl_4 system

The analysis of the ^{1}H-NMR spectra (Figure 7) of the AOT/CCl_4, MLT/CCl_4 and MLT/AOT/CCl_4 systems allows to assign AOT and MLT proton peaks.

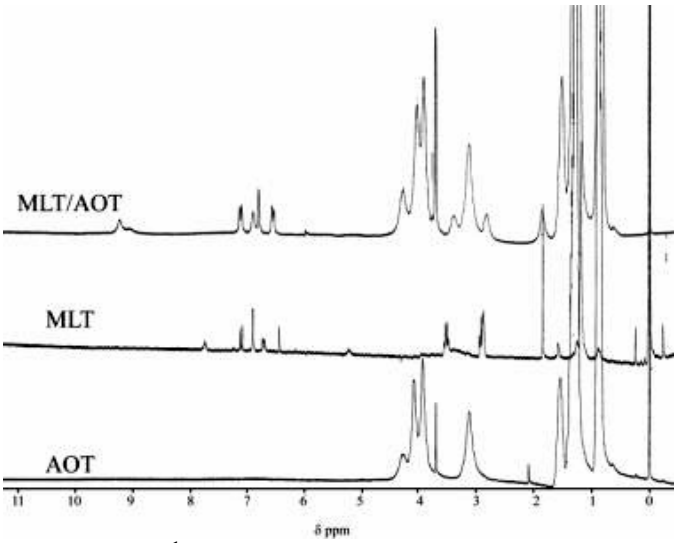


Figure 7. Comparison between the ^{1}H-NMR (CCl_4) spectra of MLT, MLT/AOT (R=0.18) and that of AOT systems.

By observing ^{1}H-NMR spectra of the MLT/AOT/CCl_4 system at the various R values, it can be noted that no R dependence of the AOT proton chemical shift occurs. This indicates that MLT interacts with AOT polar head and no significant variation of the AOT alkyl chain environment is involved. Instead, even if in smaller extent with respect to those observed for the LCT/MLT micellar systems, marked downfield shifts for both NH protons of MLT occur. The c.s. of these protons at various R values are reported in Table 2.

Table 2. Chemical shifts (ppm from TMS) of MLT indolic (a) and amidic NH (b) protons at various R

R	MLT (a)	MLT(b)
0.07	9.33	7.15
0.1	9.30	7.08
0.13	9.28	7.00
0.18	9.27	6.90

Also in this case good linear trends of the indolic and amidic NH proton chemical shifts as function of 1/R are observed (Figure 8). In particular, the chemical shifts of these protons shift towards lower fields while increasing ratio 1/R.

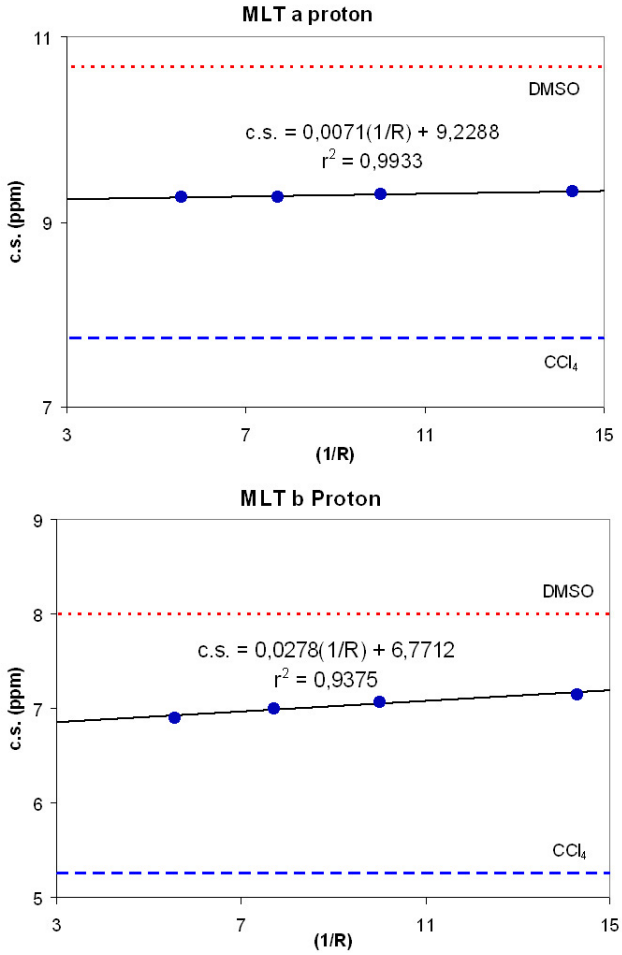


Figure 8. Chemical shift (ppm) of a and b protons of MLT confined in AOT reversed micelles as a function of 1/R. Dashed blue and dotted red lines represent the c.s. of the NH in DMSO and CCl_4, respectively.

This underlines the occurrence of some progressive variation of the H-bond strength with this parameter.

FT-IR investigation of MLT/AOT/CCl_4 system

The comparison between IR spectra of MLT dispersed in CCl_4 with that of pure MLT and MLT/AOT/CCl_4 system at various R values (Figure 9) shows that the sharp bands centered at 3460-3490 cm^{-1}, due to the indolic and amidic NH stretching of MLT monomerically dispersed in CCl_4 is shifted and broadened. This finding is consistent with the existence, in both pure MLT and MLT confined in AOT reversed micelles, of strong H-bonds involving indolic and amidic NH groups. Interestingly, comparing the position of the NH stretching band of MLT confined in AOT and in LCT, it can be argued that MLT/LCT interaction is stronger than the MLT/AOT one.

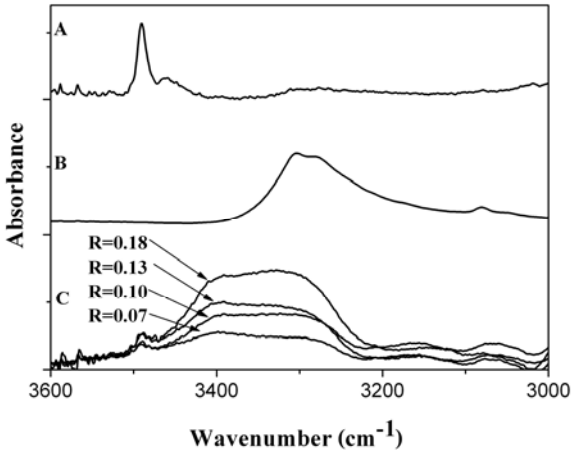


Figure 9. Comparison between FT-IR spectra of MLT/CCl_4 (A), pure MLT (B) and MLT/AOT/CCL_4 system at various R values (C).

Moreover it is worth to note that, also for the MLT/AOT/CCl_4 system, the MLT/surfactant interaction is emphasized by the monotonous shift of the surfactant SO_3^- symmetric stretching band (1052 cm^{-1}) towards lower frequencies while increasing MLT concentration in micelles (Figure 10).

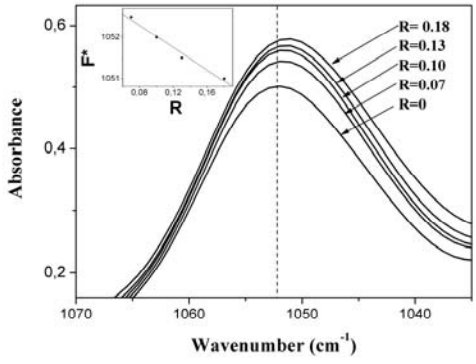


Figure 10. SO_3^- symmetric stretching bands of the MLT/AOT/CCl_4 system at various R values. In the inset is reported the frequency at the band maximum (F*) as a function of R.

This, in fact, reveals the establishment of H-bond between MLT NH protons and the surfactant anionic head.

Conclusions

By ^{1}H-NMR and FT-IR spectroscopies, information on the location and specific MLT/surfactant interactions have been emphasized. As a consequence of the formation of strong H-bonds between MLT NH and surfactant head groups, melatonin is totally entrapped in LCT and AOT reversed micelles and mainly located by opportune orientation in the nanodomain constituted by the surfactant head groups while an internal micellar core of MLT molecules is never formed even at the higher R values. Of utmost importance is the comparison between the behavior of MLT in LCT and AOT reversed micelles emphasizing the greater affinity of MLT to LCT head group as compared to AOT one. These findings suggest that, thanks to its structural features, the hormone tends to be preferentially solubilized at the interface between polar and lipophilic nanodomains allowing its antioxidant protection of hydrophilic as well as lipophilic radicals. Moreover, it can be argued that, thanks to their small size, MLT molecules can easily diffuse along polar/lipophilic interfaces reaching practically all the biological districts including water free compartments.

Experimental Section

General Procedures. Melatonin (Aldrich, 99.5%), AOT (Sigma, 99%), lecithin (Degussa, Epicuron 200; generous gift of Degussa Texturan Systems), phosphorus pentoxide (Sigma, 99%), carbon tetrachoride (Sigma, 99.97%) were used without further purification. ^{1}H-NMR spectra were recorded at 250 MHz using a Bruker AC 250 spectrometer. As internal standard it was used TMS. Shim signal was captured using DMSO as internal standard. FT- IR spectra of MLT/lecithin/CCl_4 system and MLT/AOT/CCl_4 system were recorded with a Bruker (IFS25) FT-IR spectrometer using a cell equipped with CaF_2 windows. For all measurements 100 scans in the 900-4000 cm^{-1} frequency range were carried out. All spectra were recorded at 25°C and with a spectral resolution of 1 cm^{-1}.

Lecithin/CCl_4 and AOT/CCl_4 solutions were prepared by weight at a fixed concentration of 0.1 mol/kg. To ensure the complete dehydration of these two surfactant solutions, phosphorus pentoxide was added and after stirring for 30 min. the resulting suspensions were filtered. This procedure assures the complete drying of the solutions without chemical modifications of the surfactants or composition changes of the solutions[22,23].

Then samples at various R values (R=[MLT]/[surfactant]) were prepared by adding weighed quantities of MLT to opportune quantities of the surfactant solutions. The solubilization process was sped up by ultrasonication. The complete solubilization of the substance in the surfactant solution was ascertained by visual inspection of samples maintained in a thermostatic bath at 25 °C.

Acknowledgments

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