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Effects of Curcumin Concentration and Temperature on the Spectroscopic Properties of Liposomal Curcumin

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ABSTRACT: The spectroscopic properties of liposomal curcumin in pH 7.0 sodium phosphate buffer were studied at various curcumin concentrations and temperatures. At 25 °C, liposomal curcumin exhibited much higher values than free curcumin in absorption maximum, fluorescence maximum, and fluorescence anisotropy. When curcumin concentration was increased from 2 to 20 μM, the values of fluorescence anisotropy of liposomal curcumin decreased gradually, consistent with the reduction of phase transition temperature of liposome. This observation revealed that liposomal curcumin can disrupt the packing of phospholipid bilayer and give a loose and disordered structure. On the other hand, as the temperature was increased from 25 to 80 °C, the relative intensity of maximum absorption of liposomal curcumin showed a more pronounced decrease above the phase transition temperature than lower temperatures, suggesting a weaker curcumin protection from the liquid crystalline phase of phospholipid bilayer than the rigid gel phase. However, it was observed that the fluorescence anisotropy of liposomal curcumin had higher values as the temperature increased. This phenomenon was explained as the result of location change of curcumin toward the core of phospholipid bilayer, although the structure of the phospholipid bilayer tended to be looser at higher temperatures.

KEYWORDS: *curcumin, liposome, absorption, fluorescence, anisotropy*

INTRODUCTION

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-hepadiene-3,5-dione] is a yellow lipid-soluble natural pigment extracted from the rhizome of turmeric, which is commonly used as a food colorant, spice, and cosmetic, as well as traditional medicine in Asia countries.[1,2] In the past few decades, curcumin has already become a research focus due to its numerous beneficial biological and pharmacological activities such as antioxidant,[3] antitumor,[4] anti-inflammatory,[5] and other desirable medicinal benefits.[6] Hydroxyl groups of the benzene rings, double bonds in the alkene part, and the central β-diketone moiety were suggested to play crucial roles in the beneficial activities of curcumin.[7] However, the major limitation in the application of curcumin as a health-promoting agent is reduced bioavailability.[8] One reason is that curcumin is poorly soluble in water at acidic or neutral pH, which makes curcumin hard to absorb from the gastrointestinal tract after oral administration. Another cause of low bioavailability of curcumin is due to its limited stability against gastrointestinal fluids and neutral/basic pH conditions.[9,10]

Therefore, various effective encapsulation approaches such as liposome have been designed to improve the water solubility, stability, and bioavailability of curcumin.[11] Liposome is one very promising delivery system because it has a phospholipid bilayer structure similar to that of biological membrane. Some reports have demonstrated that the employment of liposome can improve the bioactivities of curcumin, such as improved loading level in cells,[12] marked antiangiogenic effects,[13] and higher gastrointestinal absorption and plasma antioxidant activity.[11] Because of the high hydrophobicity, curcumin is thought to be loaded inside the phospholipid bilayer of the liposome. The molecular interaction between the drug and the phospholipid bilayer has decisive influence on partitioning, allocation, orientation, and conformation of the drug in the phospholipid bilayer[14,15] and thus plays an important role in the physicochemical properties and bioactivities of drug such as curcumin. However, the general relationship of encapsulated curcumin with the liposome carrier is still not well understood. Began et al. observed the nearly unchanged fluorescence anisotropy of curcumin in egg and soy liposomes and suggested that the encapsulation of curcumin almost did not change the microstructure of liposomes.[16] However, Hung et al.[17] and Barry et al.[18] recently found that binding of curcumin can induce the thinning of the phospholipid bilayer of liposomes and change microstructural properties such as the fluidity of the bilayer. On the other hand, Began et al. suggested that the equilibrium constant for the phosphatidylcholine−curcumin interaction decreased with temperature, indicating the amphiphilic nature of curcumin.[16] Kunwar et al. further concluded that curcumin is located in the gel phase of the liposomes from studies on the temperature-dependent fluorescence anisotropy of liposomal curcumin and its fluorescence quenching by acrylamide and iodide.[12] However, these reports regarding the temperature effect on the properties of liposomal curcumin were often carried out below the phase transition temperature of liposome. Because the phospholipid bilayer part of liposome where curcumin is trapped may have a phase transition from the rigid gel phase to the fluid liquid crystalline phase around the phase transition temperature,[18,19] heating is expected to significantly affect the physicochemical properties of liposomal curcumin. In addition, curcumin

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exhibits characteristic peaks in the absorption and fluorescence spectra, which can be easily monitored to follow its interaction with different carrier systems. The absorption and fluorescence methods have been utilized to understand the transport and migration of liposomal curcumin in biological systems.[12]

Taking the above into consideration, the present work is investigated the effects of curcumin concentration and temperature on the spectroscopic properties of liposomal curcumin in sodium phosphate buffer at pH 7.0. We used the thin film hydration method to prepare liposomes with 2−20 μM curcumin. Liposomes encapsulating curcumin were first studied as a function of curcumin concentration at 25 °C using UV−vis absorption and steady-state fluorescence spectra as well as fluorescence anisotropy measurements. As the temperature was increased from 25 to 80 °C, crossing the phase transition temperature of liposome, the influence of temperature on the spectroscopic properties of selected 20 μM liposomal curcumin was also investigated.

MATERIALS AND METHODS

Materials. Curcumin (lot C1386, purity ∼ 70%), L-α-phosphatidylcholine (lot P3644), and 1,6-diphenyl-1,3,5-hexatriene (DPH, lot D208000) were purchased from Sigma-Aldrich Chemical Co. As provided by the supplier, L-α-phosphatidylcholine has the following fatty acid content: 17% C16:0 (palmitic), 4% C18:0 (stearic), 9% C18:1 (oleic), 60% 18:2 (linoleic), 7% 18:3 (linolenic), and other fatty acids as minor contriutors. The phospholipid has an average of 55% (42−63%) phosphatidylcholine and 20% (10−32%) phosphatidylethanolamine. All other chemical reagents used were of analytical grade, and water was double-distilled.

Preparations of Liposomal Curcumin and Free Curcumin. The thin film hydration method was used to prepare liposomes encapsulating curcumin. Phospholipid was dissolved in the mixture solution of methanol and chloroform with the volume ratio of 1:2. Curcumin dissolved in the same mixed solvents was added into the phospholipid solution in a round-bottom flask. The organic solvents were evaporated on a rotary evaporator at 43 °C for 30 min. The dried thin phospholipid film was further maintained under a vacuum for 30 min to remove solvent traces. After the complete hydration of thin phospholipid film with a sodium phosphate buffer of pH 7.0 by first stirring for 30 min at room temperature and then incubating for 1 h at 60 °C, the liposome samples were finally obtained at a fixed phospholipid concentration (500 μM) with various curcumin concentrations (2−20 μM). These liposome samples with encapsulated curcumin did not contain nonencapsulated curcumin because there was nearly no phase separation even after 6000 rpm centrifugation. The encapsulation efficiency of liposomal curcumin is calculated to be about 45% according to the following procedure. Immediately after the end of liposome preparation, the product was centrifuged at 14000 rpm for 1 h to separate the encapsulated curcumin (sedimentation). The precipitate was dissolved in the mixed solvents of methanol and chloroform with the volume ratio of 1:2. The mass of encapsulated curcumin was analyzed according to curcumin standard curve through the measurement of maximum adsorption of curcurmin at 425 nm by UV−vis spectroscopy (Shimadzu UV-2450). The encapsulation efficiency of liposomal curcumin can be obtained from the ratio of the amount of encapsulated curcumin with that of initial added. On the other hand, free curcumin samples with various curcumin concentrations (2−20 μM) were prepared in pH 7.0 sodium phosphate buffer. A solution of curcumin dissolved in methanol was used as stock. Various small quantities of the curcumin stock solution were added to pH 7.0 sodium phosphate buffer to achieve desired curcumin concentrations.

Absorption Spectra Measurement. A series of liposomal curcumin of 2−20 μM in sodium phosphate buffer at pH 7.0 were measured in the region of 300−600 nm at 25 °C using a Shimadzu UV-2450 spectrophotometer. The absorption spectra of liposomal curcumin were compared with those of free curcumin in pH 7.0 sodium phosphate buffer. In the study of the effect of temperature on the absorption properties of encapsulated curcumin, the absorption spectra of 20 μM liposomal curcumin were recorded at 25, 30, 40, 50, 60, 70, and 80 °C, respectively. The sample of liposome encapsulating curcumin was kept at a certain temperature for 10 min before each measurement.

Steady-State Fluorescence Measurement. Steady-state fluorescence emission spectra of curcumin were measured from 450 to 750 nm with the excitation wavelength at 420 nm using an Edinburgh FLS900 spectrofluorophotometer. The fluorescence spectra of liposomal curcumin at 2−20 μM were compared with those of free curcumin in pH 7.0 sodium phosphate buffer at 25 °C. Similar to the above absorption experiments, the fluorescence spectra of 20 μM liposomal curcumin were measured at 25, 30, 40, 50, 60, 70, and 80 °C, respectively.

Fluorescence Anisotropy Technique. An Edinburgh FLS900 spectrofluorophotometer with parallel and perpendicular polarizers was used to determine the fluorescence anisotropy (r) of liposomal curcumin or 1,6-diphenyl-1,3,5-hexatriene (DPH). The fluorescence intensities were obtained at 0−0, 0−90, 90−0, and 90−90 angle settings. The value of r was calculated according to ref 20

$$r = (I_{\parallel} - G \times I_{\perp})/(I_{\parallel} + 2G \times I_{\perp})$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities of the emitted light polarized parallel and perpendicular to the exciting light, respectively, and G is the grating correction factor, which is the ratio of sensitivities of the instrument for vertically and horizontally polarized light. While liposomal curcumin was excited at 420 nm and the emission spectra were scanned from 450 to 550 nm, the fluorescence anisotropy of liposomal curcumin of 2−15 μM was measured at 25 °C, and the sample of 20 μM liposomal curcumin was measured at 25, 30, 40, 50, 60, 70, and 80 °C. When the fluorescence anisotropy of DPH encapsulated in liposome was measured at different temperatures, the excitation wavelength was set at 360 nm and the emission spectra were measured from 350 to 500 nm. The sample of liposome containing DPH was prepared using the same procedure as liposome encapsulating curcumin.

Differential Scanning Calorimetry (DSC) Measurement. Phase transition temperatures of liposomes with different curcumin concentrations of 0, 2, 5, 10, 15, and 20 μM were assayed by DSC (Setaram, Caluire, France). The liposome samples were placed in the sample cell in an approximate amount of 0.75 g. The samples were first cooled from room temperature to 10 °C for 1 h of pre-equilibration. Then the heating scans were carried out from 10 to 70 °C at a scanning rate of 1 °C/min. A baseline was recorded with sodium phosphate buffer of pH 7.0 in the reference cell and subtracted from the sample data.

RESULTS AND DISCUSSION

Curcumin is a diferuloyl methane molecule containing two ferulic acid residues joined by a methylene bridge. Because the intramolecular exciton coupling occurs between the electric dipole transition moments of two conjugated feruloyl chromophores, the low-energy $\pi-\pi^*$ excitation of the extended aromatic system is allowed.[21] Figure 1 depicts the absorption spectra of free curcumin and liposomal curcumin as a function of curcumin concentration in sodium phosphate buffer of pH 7.0 at 25 °C. Similar to the absorption features of curcumin in aqueous buffers,[16] free curcumin has an absorption peak at 427 nm together with a shoulder at 360 nm, corresponding to the absorption of conjugated diferuloyl structure and feruloyl unit, respectively.[22,23] On the other hand, the absorption curve of liposomal curcumin is characterized by a maximum absorption at 425 nm and a shoulder peak at 445 nm. With increasing curcumin concentration from 2 to 20 μM, the increases in the absorption intensities of free curcumin and liposomal curcumin

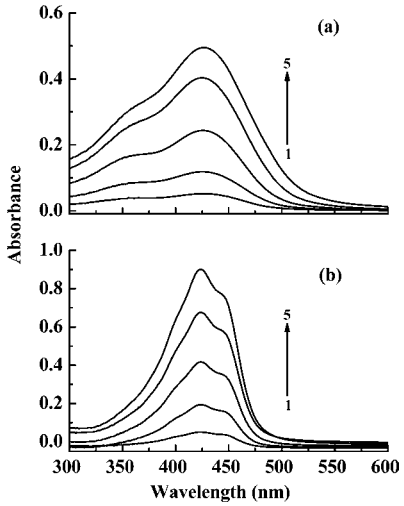

Figure 1. Absorption spectra of free curcumin (a) and liposomal curcumin (b) in pH 7.0 sodium phosphate buffer at 25 °C with various curcumin concentrations: (1) 2 μM; (2) 5 μM; (3) 10 μM; (4) 15 μM; (5) 20 μM.

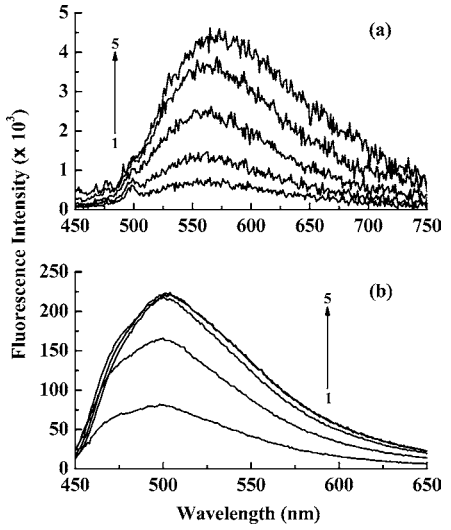

Figure 2. Fluorescence spectra of free curcumin (a) and liposomal curcumin (b) in pH 7.0 sodium phosphate buffer at 25 °C with various curcumin concentrations: (1) 2 μM; (2) 5 μM; (3) 10 μM; (4) 15 μM; (5) 20 μM.

indicate the similar concentration-dependent absorptions of curcumin in buffer and liposome. However, the observed much higher intensity of absorption maximum of liposomal curcumin with somewhat blue shift in comparison with free curcumin suggests the lower polarity of the phospholipid bilayer of liposome where curcumin is solubilized than sodium phosphate buffer.[24,25] The shoulder of free curcumin at 360 nm is due to the presence of a small population of deprotonated curcumin as a result of water−curcumin interactions in sodium phosphate buffer.[26,27] The disappeared characteristics of liposomal curcumin at 360 nm reveal that there is an associative interaction between curcumin and the phospholipid bilayer of liposome and a decrease in water−curcumin interactions, which favors the higher conjugated structure of curcumin. Additionally, the absorption peak of the $\pi-\pi^*$ transition of liposomal curcumin has a blue shift, whereas the $n-\pi^*$ transition has a red shift in the phospholipid bilayer.[28] This separation of two absorption bands of liposomal curcumin is indicated by the peak at 425 nm and the shoulder at 445 nm.

Curcumin itself is a fluorescent compound, and the fluorescence spectrum of curcumin is very sensitive to the polarity of the medium.[24,25] Figure 2 compares the emission fluorescence spectra of free curcumin and liposomal curcumin in sodium phosphate buffer of pH 7.0 at 25 °C at various curcumin concentrations. It is noted that with increasing curcumin concentration from 2 to 20 μM, fluorescence maximum often shows an increasing tendency for free curcumin at 570 nm and liposomal curcumin at 500 nm, except at curcumin concentrations of 15−20 μM for liposomal curcumin. Usually, free curcumin in sodium phosphate buffer emits relatively weak fluorescence owing to the high polarity of the medium. Nevertheless, the fluorescence maximum of liposomal curcumin shows a pronouncedly higher value and a remarkable blue shift with respect to free curcumin. This observation further confirms that the microenvironment of curcumin is changed upon liposome encapsulation; that is, curcumin moves from the polar solution to a non-polar-like bilayer part of the liposome. Additionally, the nearly coincident fluorescence curves of liposomal curcumin at 15−20 μM may be ascribed to the self-quenching of curcumin at high concentrations.[29]

Fluorescence anisotropy (r) is an experimental measure of the fluorescence depolarization mainly caused by the rotational diffusion of the fluorophore during the excited lifetime. Because the rotational mobility of encapsulated liposomal curcumin bears a close relationship to liposome, measurement of r of liposomal curcumin provides important information on the molecular interactions of curcumin with liposome. Figure 3

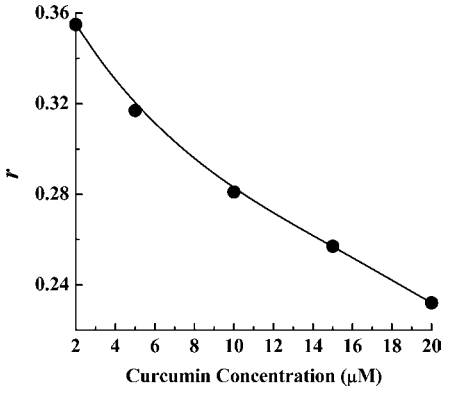

Figure 3. Variations of the anisotropy r of liposomal curcumin as a function of curcumin concentration in pH 7.0 sodium phosphate buffer at 25 °C.

shows the variation of r values of liposomal curcumin with curcumin concentration from 2 to 20 μM in sodium phosphate buffer of pH 7.0 at 25 °C. Liposomal curcumin is seen to give r values of 0.23−0.36, which is much higher than the r = 0.13 of free curcumin in aqueous solution.[24] The curcumin molecule was demonstrated to be anchored inside the phospholipid bilayer through the hydrogen bonding of −OH groups of phenolic rings of curcumin with the headgroup of phospholipid and the hydrophobic interactions of the aromatic rings of curcumin with phospholipid acyl chains.[18] These interactions of curcumin with liposome lead to a higher extent of rotation restriction of curcumin, which results in a higher r value of

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liposomal curcumin than for free curcumin. The increased r values were reported in the case of curcumin bound to proteins and surfactant micelles.[24,30] The markedly higher values of liposomal curcumin than of free curcumin in absorption maximum, fluorescence maximum, and fluorescence anisotropy indicate that liposome is a good drug delivery system, which can help to transport liposomal curcumin to desired sites in the biological systems. However, r values of liposomal curcumin present a descending trend when the curcumin concentration increases from 2 to 20 μM. The observation of lower r values at higher curcumin concentrations gives an indication that the encapsulation of curcumin in higher amount disrupts the packing of the phospholipd bilayer to a greater extent.[31]

The changes in the phase behavior of liposome upon binding of curcumin can be measured with high accuracy using DSC. Figure 4 shows DSC thermograms of liposomes with curcumin

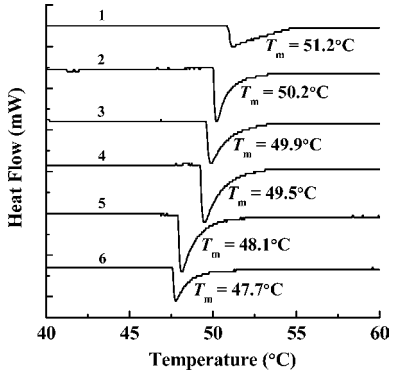

Figure 4. DSC thermograms of liposome with various curcumin concentrations: (1) 0 μM; (2) 2 μM; (3) 5 μM; (4) 10 μM; (5) 15 μM; (6) 20 μM.

concentrations from 0 to 20 μM. The endothermic peak in the DSC curve indicates the melting of the acyl chains in the phospholipid bilayer from the rigid gel phase to the fluid liquid crystalline phase. Here, the temperature of maximum endothermic peak is designated as the phase transition temperature (T_m) of liposome. It is seen that the values of T_m of liposomes encapsulating curcumin decrease gradually from 51.2 to 47.7 °C when curcumin concentration increases from 0 to 20 μM, in agreement with the literature about liposome systems.[18,19] The reduction of T_m values might indicate that the binding of curcumin perturbs the packing characteristics of the phospholipid bilayer and thus leads to a looser and more flexible structure.[32] Such influence of curcumin is a result of its structure and its molecular interaction with liposome. Curcumin is a highly conjugated and therefore rigid planar molecule. In the gel state, the presence of a rigid planar curcumin molecule can weaken hydrophobic interactions among acyl chains of phospholipids.[33] Moreover, the hydrophobic mismatch between the curcumin and the lengthier bilayer was reported to cause the bilayer to thin to match the length of the rigid curcumin.[18] Therefore, the overall effects of curcumin addition would tend to reduce the ordering of acyl chains of phospholipids, rising the fluidization of the bilayer. The change of T_m values with curcumin concentration supports our above fluorescence anisotropy results.

As shown in Figure 4, heating could lead the phospholipid bilayer of liposome to transit from the highly ordered gel phase to the fluid liquid crystalline phase. It is important to know how the temperature-induced phase change affects the spectroscopic behavior of liposomal curcumin, which is trapped in the phospholipid bilayer of liposome. Figure 5 shows the

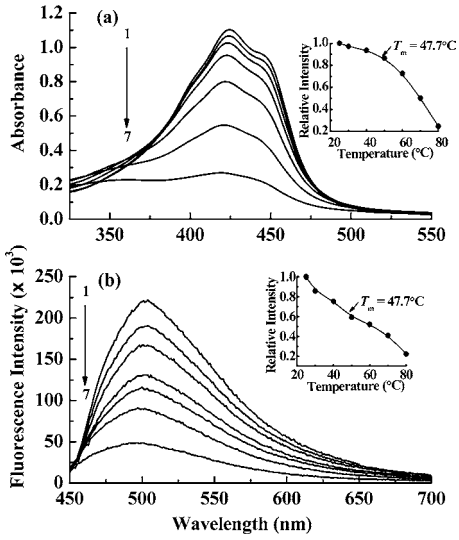

Figure 5. Absorption (a) and fluorescence (b) spectra of 20 μM liposomal curcumin in pH 7.0 sodium phosphate buffer at various temperatures: (1) 25 °C; (2) 30 °C; (3) 40 °C; (4) 50 °C; (5) 60 °C; (6) 70 °C; (7) 80 °C.

absorption and the fluorescence spectra of selected sample of 20 μM liposomal curcumin in pH 7.0 sodium phosphate buffer at 25, 30, 40, 50, 60, 70, and 80 °C, respectively. It is seen that the increase of temperature usually leads to smaller absorption and fluorescence intensities of liposomal curcumin, which indicates that increasing temperature reduces the stability of liposomal curcumin. The insets in Figure 5 further present the values of relative intensity of maximum absorption and maximum fluorescence of liposomal curcumin as a function of temperature. The phase transition temperature (T_m) of liposome with 20 μM curcumin is known to be 47.7 °C. It is worth noting that the change of relative intensity of maximum absorption of liposomal curcumin behaves differently above and below the T_m of liposome. A more pronounced decrease is seen in the values of relative intensity of maximum absorption of liposomal curcumin above T_m than below T_m. It was demonstrated that during the phase transition around T_m the phospholipid bilayer's permeability increases by several orders of magnitude due to the liposome undergoing major morphology changes,[34] including the formation of open liposome, bilayer disks, and pore-like defects. As a result, more fluid liquid crystalline phase of phospholipid bilayer has a weaker curcumin protection than the rigid gel phase, leading to a fast decrease of the relative intensity of maximum absorption of liposomal curcumin above T_m. Nevertheless, the decreasing rate of the relative intensity of maximum fluorescence seems to not have much relationship with the T_m of liposome encapsulating curcumin. This result may be ascribed to the generally dominant heating-reduced quantum yield of the fluorophores due to the increased rate of oxygen diffusion.[35] We thus cannot clearly see the influence of the phase transition of the phospholipid bilayer on the change of fluorescence intensity of liposomal curcumin upon heating.

To further investigate the effect of heating on the microproperties of liposomal curcumin, we have carried out measurements of the fluorescence anisotropy (r) of DPH and

curcumin encapsulated in liposome for comparison at various temperatures. Figure 6 presents the changes of r values of

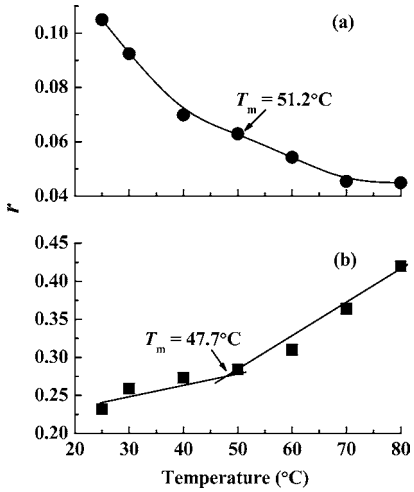

Figure 6. Variations of the anisotropy r of liposomal DPH (a) and liposomal curcumin (b) as a function of temperature in pH 7.0 sodium phosphate buffer.

liposomal DPH and liposomal curcumin as a function of temperatures. As a widely used fluorescent anisotropy probe, DPH with high hydrophobicity is generally thought to be located parallel to the phospholipid acyl chain axis and buried near the hydrophobic core in the phospholipid bilayer.[36] Therefore, the r values of DPH are mostly sensitive to the structure change of the phospholipid bilayer of liposome.[37] It is seen that the r values of DPH decrease gradually as the temperature rises, which is a general observation in the literature regarding the heat-enhanced fluidity of the phospholipid bilayer of liposome. Because heating leads the phospholipid bilayer of liposome to transit from the highly ordered gel phase to the fluid liquid crystalline phase around the phase transition temperature, there are smaller r values of DPH above T_m than below.

Opposite from the changes of DPH, liposomal curcumin exhibits increased r values at higher temperatures. This interesting phenomenon may be mainly ascribed to the location change of curcumin in liposomes. Although the curcumin molecule is anchored inside the phospholipid bilayer, the polar portions of curcumin let the curcumin molecule be located closer to the interfacial region of the bilayer than DPH.[19] With increasing temperature, the phospholipid bilayer goes into a looser state, but the curcumin molecule with rigid planar structure is ready to penetrate more deeply inside the bilayer,[33] which can stiffen the packing of the bilayer. This effect is more pronounced at the fluid liquid crystalline phase of the phospholipid bilayer than at the highly ordered gel phase, as indicated by the faster increase in r values of liposomal curcumin above T_m than below as shown in Figure 6b. In addition, one drawback associated with the use of liposome as drug delivery vehicle is the susceptibility to undergo leakage of entrapped drug during temperature increase. However, the increased r value of liposomal curcumin suggests that encapsulated curcumin is likely to stay at a deep location of the phospholipid bilayer even at high temperatures, which may promote the absorption of curcumin in cellular and gastrointestinal systems.

In conclusion, the encapsulation of curcumin in liposome brings about markedly higher absorption maximum, fluorescence maximum, and fluorescence anisotropy than that free curcumin. Upon increasing curcumin concentration, the changes of values of fluorescence anisotropy of liposomal curcumin and the phase transition temperature of liposome reveal looser and more disordered structure of the phospholipid bilayer induced by encapsulated curcumin. However, as the temperature increases, the fluorescence anisotropy of liposomal curcumin shows an increasing tendency, which is ascribed to the moving of curcumin toward the core of phospholipid bilayer. Thus, the current work discloses that curcumin concentration and temperature influence the spectroscopic properties of liposomal curcumin and the microstructure of phospholipid bilayer in different ways.

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Notes

The authors declare no competing financial interest.

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