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ARTICLE *in* THE JOURNAL OF PHYSICAL CHEMISTRY B · FEBRUARY 2011

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Cooperative Binding and Stabilization of the Medicinal Pigment Curcumin by Diamide Linked γ-Cyclodextrin Dimers: A Spectroscopic Characterization

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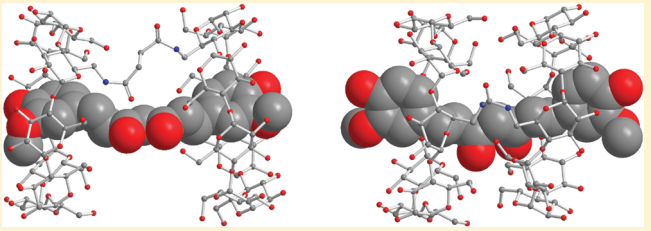
Supporting Information

ABSTRACT: Diamide linked γ-cyclodextrin (γ-CD) dimers are used to capture curcumin and suppress its decomposition in water. In this study, succinamide and urea linked γ-CD dimers joined through the $C6^A$ carbon on each γ-CD are used. The γ-CD dimers, $66\gamma CD_2su$ and $66\gamma CD_2ur$, show a remarkable ability to suppress the decomposition of curcumin and extend its half-life from less than 30 min to greater than 16 h. The 1:1 association of curcumin with $66\gamma CD_2su$ and $66\gamma CD_2ur$ has high stability constants of 8.7×10^6 M^{-1} and 2.0×10^6 M^{-1}, respectively. In addition, 2D ^1H NOESY NMR results show specific hydrogen interactions in the association of curcumin with $66\gamma CD_2su$ and $66\gamma CD_2ur$, consistent with the cooperative binding of curcumin by both γ-CD annuli of $66\gamma CD_2su$ and $66\gamma CD_2ur$. The interactions between curcumin in the linked γ-CD dimers and surfactant micelles were studied using fluorescence spectroscopy. While linked γ-CD dimer-bound curcumin has a negligible fluorescence quantum yield, a significant increase in fluorescence intensity ($\Phi_{fl} > 2\%$) in the presence of micelles suggests that curcumin is delivered to the micelle. The overall results indicate that the diamide linked γ-CD dimers are highly promising systems for curcumin delivery in vivo due to effective curcumin stabilization.



INTRODUCTION

Curcuminoids are a group of yellow polyphenols present in the rhizomes of the spice plant *curcuma longa*, commonly known as turmeric. The most abundant form is curcumin (Figure 1), which constitutes 77% of curcuminoids in company with demethoxycurcumin (17%) and bisdemethoxycurcumin (3%). In addition, another curcuminoid, cyclocurcumin, which was isolated in 1993, is present at a low level.[1] The health and medicinal effects of curcuminoids have been documented in ancient Indian literature.[2] In the past decade, scientific studies have shown that the medicinal effects of curcumin include anti-inflammatory,[3] anticancer,[4,5] anti-Alzheimer's,[6] anticystic fibrosis,[7] and wound healing properties.[8,9] As a result, several clinical trials are either underway or have been completed with an aim to develop curcumin into a treatment agent.[10,11]

From the chemical standpoint, there are two challenges that must be overcome in order for curcumin to become a routine treatment agent. First, curcumin has a low aqueous solubility which limits its availability in vivo. Second, for the limited concentration that is soluble, curcumin is prone to undergoing hydrolysis, which results in substantial degradation within 30 min.[12,13] Methods of encapsulation and delivery have been developed to address these two challenging issues which include using molecular assemblies such as micelles,[14−16] globular proteins,[12,17,18] and polymer nanoparticles.[19−21] While these methods are highly effective in enabling curcumin to be present in aqueous solutions in up to mM concentration and inhibiting degradation almost entirely, the large size of such assemblies may affect intracellular curcumin delivery. Moreover, these curcumin encapsulation species typically experience significant perturbation in their structures upon contact with biomembranes, releasing the encapsulated agent instead of entering the membrane intact. Therefore, development of smaller sized encapsulation species is of considerable interest as they may remain intact and be the basis of a highly effective delivery method.

Cyclodextrins (CDs) are naturally occurring cyclic oligosaccharides with either 6 (α-), 7 (β-), or 8 (γ-) glucopyranoside units. They are often used as stabilizers and carriers for drugs due to their annular structure which has a hydrophobic interior and a hydrophilic exterior.[22] This characteristic of cyclodextrins allows hydrophobic drugs to be solubilized in water in a relatively stable condition. In order to further improve the drug-carrier binding to increase drug-carrier ratio, strategies involving cooperative binding of a cyclodextrin host and a molecular guest have been

Received: October 7, 2010
Revised: December 6, 2010
Published: December 31, 2010

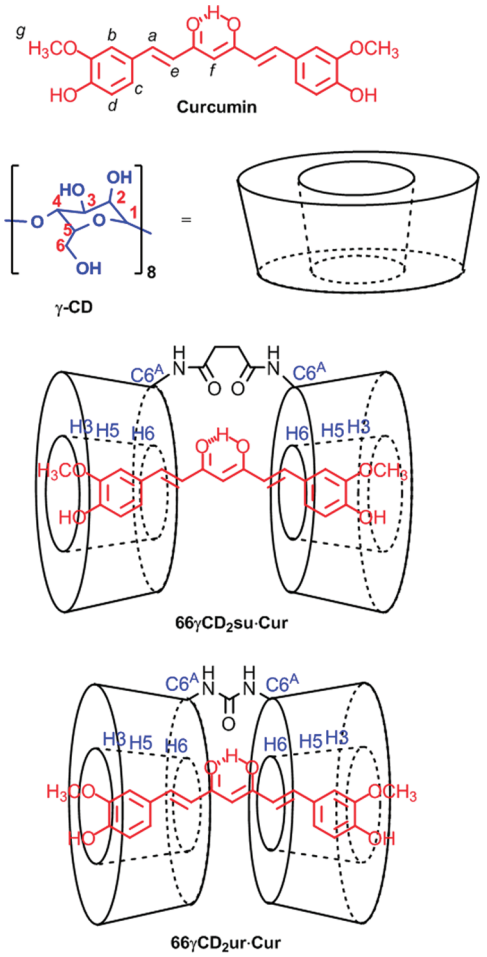


Figure 1. Structures of curcumin, γ-CD and curcumin in 66γCD$_2$su and 66γCD$_2$ur. The hydrogens of curcumin and γ-CD are labeled as a−f and 1−6, respectively. All species are drawn to scale in this figure.

developed.[23,24] In particular, linked cyclodextrins have been synthesized and used in cooperative binding with a range of guests.[25−28] Recently, Pham et al. reported the synthesis of diamide linked γ-cyclodextrin (γ-CD) dimers, which have considerable potential as effective drug carriers.[29] The diamide linker is expected to be hydrolyzed enzymatically in the intracellular environment, thereby releasing the encapsulated materials.[30−33]

In this article, we report the stabilization effect of two diamide linked γ-CD dimers on curcumin degradation in the aqueous environment at pH 7.4 and 37 °C. The linked γ-CD dimers with either a succinamide or a urea linker show an impressive ability to suppress degradation of curcumin in aqueous solution. The 2D ^{1}H NOESY NMR results indicate that the binding of curcumin by the linked γ-CD dimers is cooperative, as shown in Figure 1. The potential for drug delivery applications of the linked γ-CD dimers was also investigated. The increase of fluorescence quantum yield from nearly zero in water to ~2% in a micellar solution indicates that the linked γ-CD dimer-curcumin complex has a strong affinity to micelles. In short, the cooperative binding between curcumin and diamide linked γ-CD dimers has been assessed with spectroscopic techniques and the linked γ-CD dimer-bound curcumin exhibits a highly suppressed degradation. As a result, diamide linked γ-CD dimers show significant promise as effective delivery agents for curcumin.

EXPERIMENTAL SECTION

Materials. Curcumin was obtained from LKT Laboratories (purity >98%) and Sigma Aldrich (70% curcumin and 30% demethoxy- and bisdemethoxycurcumin). Curcumin from the first source was used in the 2D ^{1}H NOESY NMR studies and the remaining spectroscopic studies were performed with the product from the second source. Our previous study showed that curcumin from the second source was suitable for spectroscopic investigations.[34] Sodium dodecyl sulfate, SDS, (≥99.0% by GC assay) was purchased from Fluka. Methanol (AR grade, 99.5%) from Merck Pty Ltd. was used as received. The α-, β-, and γ-CD were purchased from Nihon Shokuhin Kako Co. without further purification. The C6^A-to-C6^A diamide linked γ-CD dimers, *N,N′*-bis(6^A-deoxy-γ-cyclodextrin-6^A-yl) succinamide and *N,N′*-bis(6^A-deoxy-γ-cyclodextrin-6^A-yl) urea, which are abbreviated as 66γCD$_2$su and 66γCD$_2$ur, respectively, were synthesized using methods established by Pham et al.[29] In addition to γ-CD, other C6^A-to-C6^A diamide linked CD dimers were also synthesized with β-CD to produce 66βCD$_2$su and 66βCD$_2$ur according to established methods.[35] Phosphate buffer solutions (50 mM) used in this study were prepared with water from a Millipore Milli-Q NANO pure water system and the pH was adjusted to 7.4 with HCl and NaOH.

UV−Visible Absorption and Fluorescence Spectra of Curcumin. All experiments were performed under physiological conditions (pH 7.4 phosphate buffer, 37 °C). A solution of 1 mg/mL curcumin in methanol was used as a stock solution, 16.61 μL of which was added to 3 mL of the phosphate buffer solution without CD to give [curcumin] of 15 μM. Solutions of 1 mM α-, β- and γ-CD were also prepared in phosphate buffer solution. Curcumin stock solution (11.07 μL) was added to 3 mL of each of 1 mM α-, β- and γ-CD solutions to achieve 1:100 mol ratio of curcumin to each CD with a final [Cur] = 10 μM. Similarly, stock solutions of the diamide linked γ-CD dimers in water were prepared with [66γCD$_2$su] = 2.50 mM and [66γCD$_2$ur] = 2.22 mM, and they were diluted to 15 μM with phosphate buffer solution. Curcumin stock solution (16.61 μL) was added to 3 mL of each of 15 μM the diamide linked γ-CD dimer solutions to achieve a 1:1 mol ratio of curcumin to each diamide linked γ-CD dimer with a final [Cur] = 15 μM. Absorbance readings were taken from 300 to 700 nm using a Varian Cary 5000 UV−vis/NIR spectrophotometer. In the experiments where degradation of curcumin in the buffer solution was recorded, the UV−vis absorption spectra were collected over 30 min at 1-min intervals. However, for the experiments where degradation of curcumin in α-, β-, and γ-CD and diamide linked γ-CD dimer solutions were investigated, the UV−vis absorption spectra were collected over 15 h at 30-min intervals. Fluorescence spectra of curcumin in 66γCD$_2$su and 66γCD$_2$ur solutions (1.73 μM and 1.63 μM, respectively) at 1:1 mol ratio with and without SDS micelles were recorded from 390 to 740 nm using a Varian Cary Eclipse Fluorescence spectrophotometer with the excitation and emission slit widths set at 5 nm. The excitation wavelength for these experiments was set at 380 nm. The SDS concentration was kept at 16.2 mM, which is twice the critical micelle concentration. For fluorescence quantum yield measurements, Rhodamine B was used as a reference (Φ_{fl} = 31%) with an excitation wavelength of 530 nm.[36] The Φ_{fl} of 66γCD$_2$su-curcumin and 66γCD$_2$ur-curcumin complexes were calculated based on the fluorescence quantum yield of Rhodamine B using established methods.[37]

Stability Constants of 66γCD$_2$su−Curcumin and 66γCD$_2$ur−Curcumin Complexes. In the UV−vis titration experiment, a small quantity of 0.125 mg/mL stock solution of curcumin in methanol was added in a stepwise fashion to a 3-mL solution of 15 μM 66γCD$_2$su or 66γCD$_2$ur to achieve a series of curcumin concentrations from 8 to 20 μM. Within this range of concentrations, aggregation of curcumin appeared to be negligible. All experiments were performed in less than 60 min under physiological conditions (pH 7.4, 37 °C in phosphate buffer). The model used for determining the stability constant assumes that curcumin exists in the free form (Cur) and in the 1:1 host−guest complexed form (66γCD$_2$su·Cur) characterized by different molar absorptivities, as exemplified for 66γCD$_2$su in eq 1

$$66\gamma CD_2su + Cur \overset{K}{\rightleftharpoons} 66\gamma CD_2su \cdot Cur \quad (1)$$

The stability constant, K, at equilibrium is given by

$$K = [66\gamma CD_2su \cdot Cur]/[66\gamma CD_2su][Cur] \quad (2)$$

Given that $[Cur]_0$ and $[66\gamma CD_2su]_0$ are the initial concentrations of the two complexation partners

$$[Cur]_0 = [66\gamma CD_2su \cdot Cur] + [Cur] \quad (3)$$

$$[66\gamma CD_2su]_0 = [66\gamma CD_2su \cdot Cur] + [66\gamma CD_2su] \quad (4)$$

The measured absorbance at a particular wavelength is given by

$$A(\lambda) = \varepsilon(\lambda)[Cur]_0$$
$$= \varepsilon_{Cur}(\lambda)[Cur] + \varepsilon_{66\gamma CD_2su \cdot Cur}(\lambda)[66\gamma CD_2su \cdot Cur] \quad (5)$$

where ε, ε_{Cur}, and $\varepsilon_{66\gamma CD_2su \cdot Cur}$ represent the apparent molar absorptivity and molar absorptivities of Cur and 66γCD$_2$su·Cur, respectively, and the measurement cell length was 1 cm. The value of ε_{Cur} measured with [Cur] = 20 μM from the wavelength range 350−500 nm at 0.5 nm intervals was used as an input to the model. The reference solutions in the experiments contained the same 66γCD$_2$su or 66γCD$_2$ur concentration as the sample solutions. The values of K and $\varepsilon_{66\gamma CD_2su \cdot Cur}$ were obtained by solving eqs 2−5 which were best fitted to the data using a nonlinear least-squares program (HypSpec).[38,39]

2D ^{1}H NOESY NMR Spectra of Curcumin Complexes. The 2D ^{1}H NOESY NMR spectra of curcumin complexes with 66γCD$_2$su and 66γCD$_2$ur were recorded with a Varian-Inova 600 spectrometer operating at 599.602 MHz using a standard pulse sequence with a mixing time of 300 ms and an acquisition time of 150 ms with 8 repetitions. Volumes of 7.15 and 7.30 μL of a 40 mg/mL stock solution of curcumin in d_6-DMSO were added to 0.7 mL of 3 mg/mL solutions of 66γCD$_2$su and 66γCD$_2$ur in D$_2$O, respectively, to achieve curcumin concentrations of 1.11 mM and 1.13 mM, which resulted in a 1:1 mol ratio of curcumin to 66γCD$_2$su or 66γCD$_2$ur. The resultant curcumin concentration was approximately 417 μg/mL, which is significantly higher than the aqueous solubility of curcumin,[40] consistent with 66γCD$_2$su and 66γCD$_2$ur enhancing the aqueous solubility of curcumin.

RESULTS AND DISCUSSIONS

Degradation of Curcumin in Phosphate Buffer and Suppression of Degradation by γ-CD. The effect of association with CDs on the degradation of curcumin was investigated.

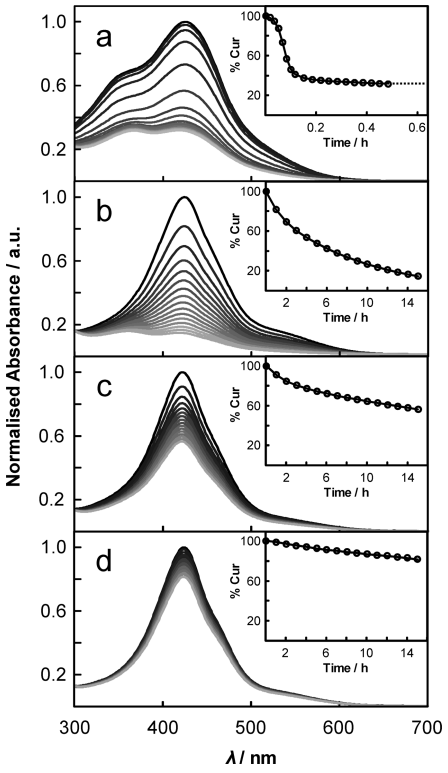


Figure 2. UV−vis absorption spectra of curcumin in (a) pH 7.4 phosphate buffer alone, (b) γ-CD, (c) 66γCD$_2$su, and (d) 66γCD$_2$ur at 37 °C. The ratios of curcumin to γ-CD, 66γCD$_2$su, and 66γCD$_2$ur are 1:100, 1:1, and 1:1, respectively. In (a), (c), and (d), [curcumin] = 15 μM but only 10 μM in (b). Signals were recorded for 30 min in (a) but 15 h in (b), (c), and (d). The insets show the decay at the absorption maxima (430 nm) due to degradation.

Figure 2a shows the UV−vis absorption spectrum of curcumin in phosphate buffer, in which a peak around 430 nm and a shoulder at 350 nm are observed. These spectroscopic features are in agreement with our previous results.[12,41] In the current study, the UV−vis absorption spectra of curcumin in phosphate buffer were recorded for 30 min at 1-min intervals and the time dependent spectra are shown in Figure 2a. Curcumin shows a high level of degradation in the 30-min period, and the decay of the absorption maximum is shown in the inset of Figure 2a. The results show that curcumin undergoes rapid degradation in the absence of CDs, giving rise to a half-life of 0.09 ± 0.04 h. Recent work has established that curcumin exists in solution in the keto−enol tautomeric form (Figure 1).[42] Under alkaline conditions, the enol group of curcumin becomes deprotonated and it is likely to undergo degradation by a retro-aldol condesation reaction catalyzed by the hydroxide anion.[43] The degradation process leads to fragmentation of curcumin to form *trans*-6-(4′-hydroxy-3′-methoxyphenyl)-2,4-dioxo-5-hexenal, vanillin, ferulic acid, and feruloyl methane.[13,43]

The UV−vis absorption spectrum of curcumin in γ-CD is shown in Figure 2b. Curcumin shows an absorption peak at 430 nm in the presence of γ-CD and the spectrum is clearly different from that of curcumin in phosphate buffer alone. In particular, the shoulder at 350 nm that is found in the spectrum of curcumin in phosphate buffer is noticeably missing in that of curcumin in the presence of γ-CD. It has been established that the 350-nm spectral shoulder is a result of water-curcumin interactions.[12,41]

Therefore, the lack of this spectral shoulder implies that there is an associative interaction between curcumin and γ-CD and a decrease in water-curcumin interaction, which is supported by the results on degradation of curcumin, as follows. Figure 2b shows the UV−vis absorption spectra of curcumin (10 μM) in the presence of γ-CD (1 mM) taken over a period of 15 h at 1 h intervals and the inset shows the progressive decrease of absorbance at 430 nm. The results show that binding of curcumin by γ-CD plays a role in suppression of degradation of curcumin. Although curcumin degrades in phosphate buffer alone to a baseline level in less than 30 min (Figure 2a), the degradation in the presence of γ-CD is significantly suppressed such that approximately 10% of curcumin is still present after 15 h. The half-life of the degradation is 4.46 ± 0.12 h, which is roughly 50 times longer than that of curcumin in phosphate buffer alone. In addition to γ-CD, both α- and β-CD also demonstrate an ability to suppress degradation of curcumin at 1 mM concentration (Supporting Information). However, the level of suppression of degradation is substantially less than that caused by γ-CD, which suggests that the binding between curcumin and α-CD and β-CD is relatively weak.

It is important to stress that a substantially higher concentration of γ-CD than curcumin is required to result in the degree of suppression of degradation shown in Figure 2b. In this case, [γ-CD] is 100 times higher than [curcumin]. The requirement for a high concentration suggests that the binding between γ-CD and curcumin is also weak.

Suppression of Curcumin Degradation in the Presence of 66γCD$_2$su and 66γCD$_2$ur. Figure 2, panels c and d, shows the UV−vis absorption spectra of curcumin in the presence of 66γCD$_2$su and 66γCD$_2$ur, respectively. The structures of 66γCD$_2$su and 66γCD$_2$ur (Figure 1) show that the narrow ends of γ-CDs are linked. Similar to the case of γ-CD, the absence of the spectral peak at 350 nm implies that there is an association between curcumin and 66γCD$_2$su and 66γCD$_2$ur as is discussed below. The degradation results indicate that a significant portion of curcumin is still stable after 15 h. The kinetics of degradation are shown in the insets and the half-lives of curcumin in 66γCD$_2$su and 66γCD$_2$ur are estimated to be 16.50 ± 2.61 and 70.67 ± 19.20 h, respectively, which are approximately 180 and 780 times longer than that in phosphate buffer alone. These half-lives are remarkably long especially since only 15 μM of either 66γCD$_2$su or 66γCD$_2$ur were used to stabilize 15 μM of curcumin in a 1:1 host-guest ratio. This high level of stabilization of curcumin by a relatively low concentration of either 66γCD$_2$su or 66γCD$_2$ur is in sharp contrast to the effect of γ-CD alone, which requires a concentration ratio of 100:1 in order to achieve a similar level of stabilization. As is discussed below, cooperative binding of curcumin by either 66γCD$_2$su or 66γCD$_2$ur leads to an effective suppression of degradation.

The origin of the greater ability of 66γCD$_2$ur than 66γCD$_2$su to stabilize curcumin can be deduced by considering the availability of water in the vicinity of the linker. As mentioned above, hydrolysis is involved in the degradation of curcumin. The succinamide linker of 66γCD$_2$su is approximately twice the length of the urea linker of 66γCD$_2$ur (Figure 1). Although water is largely excluded from the linked dimers, given the length of the succinamide linker, the keto−enol group of a 66γCD2su-bound curcumin is likely to be more exposed to water, increasing the probability of undergoing a retro-aldol hydrolysis reaction. In contrast, the short and less flexible urea linker in 66γCD$_2$ur results in a narrower gap between the two γ-CD units, which

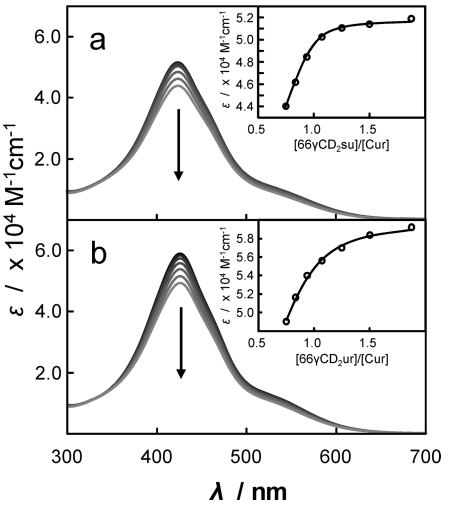


Figure 3. Apparent molar absorptivity (ε) spectra of curcumin in titration studies in phosphate buffer at pH 7.4, 37 °C. Curcumin was added in a stepwise fashion to 15 μM of (a) 66γCD$_2$su and (b) 66γCD$_2$ur to achieve a series of curcumin concentrations from 8 to 20 μM within 60 min. The insets show binding curves of curcumin in 66γCD$_2$su and 66γCD$_2$ur, respectively, which correspond to stability constants of (8.7 ± 0.4) × 10^6 and (2.0 ± 0.1) × 10^6 M^{-1}.

significantly limits the access of water to the keto-enol group of curcumin. Moreover, the proximity between the annuli enables the keto-enol group of curcumin to be partially protected by the γ-CDs of 66γCD$_2$ur (Figure 1), leading to a more effective stabilization of curcumin than 66γCD$_2$su.

Stability Constants of 66γCD$_2$su−Curcumin and 66γCD$_2$ur−Curcumin Complexes. The variation of the absorptivity of curcumin in the presence of 66γCD$_2$su (Figure 3a) is consistent with the formation of a dominant 1:1 66γCD$_2$su:Cur host−guest complex. The best fit using a model for the formation of this complex (Experimental Section) to the variation of molar absorptivity as a function of total [Cur] was obtained in the range 350−500 nm at 0.5 nm intervals. Analysis of the results yielded a stability constant (K) of (8.7 ± 0.4) × 10^6 M^{-1} with λ_{max} = 424 nm and $\varepsilon_{66\gamma CD_2su\cdot Cur}$ = 5.21 × 10^4 M^{-1} cm^{-1} for curcumin in the 66γCD$_2$su·Cur complex (Supporting Information). In comparison, free curcumin exhibits λ_{max} = 426 nm and ε_{Cur} = 2.16 × 10^4 M^{-1} cm^{-1}. The variation of the molar absorptivity at 424 nm and the best-fit curve of 66γCD$_2$su·Cur are shown in the inset of Figure 3a. Similar variations in molar absorptivity occur for the 66γCD$_2$ur·Cur complex for which K = (2.0 ± 0.1) × 10^6 M^{-1} with λ_{max} = 425 nm ($\varepsilon_{66\gamma CD_2ur\cdot Cur}$ = 6.15 × 10^4 M^{-1} cm^{-1}) were obtained (Figure 3b). Interestingly, the K value of the 66γCD$_2$su·Cur complex is higher than that of the 66γCD$_2$ur·Cur complex, even though 66γCD$_2$ur exhibits a greater ability to suppress degradation of curcumin in phosphate buffer. However, as is discussed below, the higher K value for the association of curcumin with 66γCD$_2$su than with 66γCD$_2$ur correlates with a higher level of hydrogen−hydrogen van der Waals interactions, as shown using 2D ^{1}H NOESY. The high K value of the 66γCD$_2$su-curcumin complex is likely to arise from the van der Waals interactions between the hydrophobic γ-CD annuli and the aromatic groups of curcumin. However, due to the shorter linker of 66γCD$_2$ur, the spatial overlap between γ-CD and the aromatic groups of curcumin is less effective, which in turn results in a lower K value. In addition to van der Waals

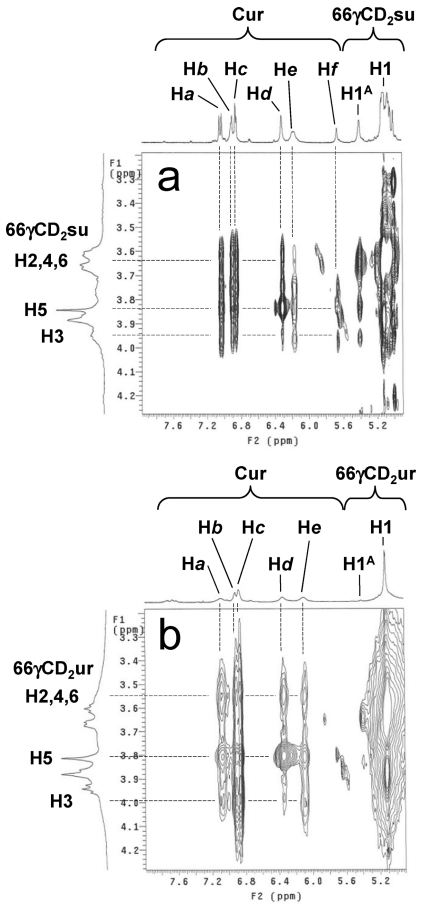


Figure 4. 2D NOESY NMR spectra of curcumin in (a) 66$\gamma$$CD_2$su and (b) 66$\gamma$$CD_2$ur in D_2O. The mole ratio of curcumin:66$\gamma$$CD_2$su or 66$\gamma$$CD_2$ur is 1:1, with a concentration of approximately 1.1 mM. Interactions between the hydrogens of curcumin and those of the γ-CD moieties are indicated by dashed lines.

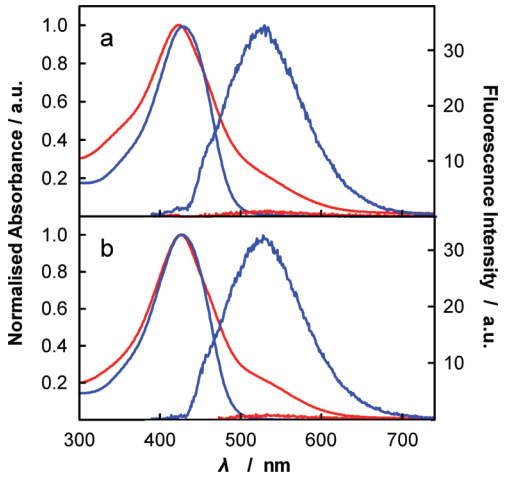


Figure 5. UV−vis absorption and fluorescence spectra ($\lambda_{excitation}$ = 380 nm) of curcumin in the presence of (a) 66$\gamma$$CD_2$su and (b) 66$\gamma$$CD_2$ur with (blue) and without (red) SDS micelles in pH 7.4 phosphate buffer solution at 37 °C. The mole ratio of curcumin to 66$\gamma$$CD_2$su (1.73 μM) or 66$\gamma$$CD_2$ur (1.63 μM) is 1:1. The concentration of SDS is 16.2 mM.

interactions, the keto−enol group of curcumin is capable of forming hydrogen bonds with the diamide linkers of 66$\gamma$$CD_2$su and 66$\gamma$$CD_2$ur and their primary hydroxy groups, which may also contribute to the association of curcumin with either 66$\gamma$$CD_2$su or 66$\gamma$$CD_2$ur. It is interesting that the magnitude of K is not directly proportional to the half-life of curcumin degradation. It is important to note that the time scale of degradation depends strongly on limiting the access of water to curcumin, particularly at the keto−enol moiety. As mentioned above, 66$\gamma$$CD_2$ur appears to have a greater ability to exclude water from the keto−enol group of curcumin.

2D ^{1}H NOESY NMR Studies of 66$\gamma$$CD_2$su−Curcumin and 66$\gamma$$CD_2$ur−Curcumin Complexes. The 2D ^{1}H NOESY NMR technique was employed to provide insight into the structures of the 66$\gamma$$CD_2$su-curcumin and 66$\gamma$$CD_2$ur-curcumin complexes. The spectra of D_2O solutions of curcumin in 66$\gamma$$CD_2$su and in 66$\gamma$$CD_2$ur at 1:1 mol ratio are shown in Figure 4. In these spectra, the presence of prominent cross-peaks, as highlighted by dashed lines, is a clear indication of hydrogen interactions between curcumin and the diamide linked γ-CD dimers, which are discussed below. The assignment of the 2D ^{1}H NOESY NMR spectra in Figure 4 is facilitated by the well characterized ^{1}H NMR spectra of curcumin, 66$\gamma$$CD_2$su and 66$\gamma$$CD_2$ur (Supporting Information).[44] The labeling of the hydrogens of curcumin, 66$\gamma$$CD_2$su and 66$\gamma$$CD_2$ur are shown in Figure 1. The ^{1}H NMR signals of 66$\gamma$$CD_2$su and 66$\gamma$$CD_2$ur in the presence of curcumin exhibit minor differences from those without curcumin. However, the signals of curcumin arising from Ha to Hf show a clear upfield shift of approximately 0.5 ppm relative to those in the absence of the diamide linked γ-CD dimers (Supporting Information). The upfield shift of the peak which corresponds to the methoxy groups (Hg) of curcumin cannot be assigned definitively due to an overlap with the H2−6 peaks of 66$\gamma$$CD_2$su and 66$\gamma$$CD_2$ur but it also appears to be close to 0.5 ppm. In addition to the cross-peaks, this upfield shift of the curcumin ^{1}H NMR signals serves as an indication of a change in the magnetic environment of curcumin due to complexation by either 66$\gamma$$CD_2$su or 66$\gamma$$CD_2$ur.

The 2D ^{1}H NOESY NMR spectrum of curcumin in 66$\gamma$$CD_2$su (Figure 4a) shows cross-peaks arising from strong interactions of the phenyl, trans, and alpha hydrogens (Ha−Hf) of curcumin with all of the hydrogens (H3, H5−6) in the interior of the γ-CD annulus, as illustrated by the series of dashed lines in Figure 4a. These results indicate strong binding between curcumin and 66$\gamma$$CD_2$su. The $H1^A$ signal of 66$\gamma$$CD_2$su is shifted downfield from the signals of the other H1 hydrogens which show a well-resolved multiplet. This multiplet arises because of the substitution of the succinamide linker onto the $C6^A$ carbon of the A glucopyranose unit of each γ-CD. In addition, a similar but lesser chemical shift differentiation of the H1 hydrogens of the seven adjacent but unsubstituted glucopyranose units B−G also occurs. However, the differentiation between the H1 signals of these seven units observed in the presence of curcumin is greater than that observed in its absence,[29] which suggests that the 66$\gamma$$CD_2$su-curcumin interactions increase this chemical shift differentiation. A similar situation appears to arise with the 66$\gamma$$CD_2$su H3−H6 hydrogen signals such that the signals from each is spread over a frequency range on the F1 axis in Figure 4a and the labeling is consequently indicative only. As a result, the cross-peaks arising from curcumin Ha−Hf hydrogens with 66$\gamma$$CD_2$su H3, H5, and H6 are extended over a substantial range on the F1 axis in Figure 4a.

Table 1. Host-Curcumin Mole Ratio, Half-Life of Degradation, Yield of Suppression, Stability Constant, and Fluorescence Quantum Yield at pH 7.4 and 37 °C

host	solvent	host-curcumin mole ratio	half-life (h)	stability constant (M^{-1})	fluorescence quantum yield (%)
	PBS[a]		0.09 ± 0.04		~0
γ-CD	PBS	100:1	4.46 ± 0.12	*b*	~0
66γCD_2su	PBS	1:1	16.50 ± 2.61	(8.7 ± 0.4) × 10^6	0.06 ± 0.01
66γCD_2su	PBS + SDS micelle	1:1	*b*	*b*	2.47 ± 0.18
66γCD_2ur	PBS	1:1	70.67 ± 19.20	(2.0 ± 0.1) × 10^6	0.04 ± 0.01
66γCD_2ur	PBS + SDS micelle	1:1	*b*	*b*	2.07 ± 0.27

[a] Phosphate buffer solution (50 mM). [b] Not determined in this study.

The 2D ^{1}H NOESY NMR spectrum shown in Figure 4b indicates that there is strong binding of curcumin to 66γCD_2ur because of the high intensity of cross-peaks as well as broadening of the peaks of curcumin. The spectrum shows cross-peaks arising from strong interactions of the H*a*−H*e* of curcumin with the annular hydrogens (H3, H5−6) of 66γCD_2ur. In particular, the H*e* peak of curcumin shows stronger interaction with H6 of 66γCD_2ur than 66γCD_2su, indicating the relative proximity of these two hydrogens. This observation is consistent with the structure in Figure 1, the diamide linkage of 66γCD_2ur being shorter than that of 66γCD_2su.

The H*a*−H*f* signals of curcumin in the presence of 66γCD_2su and those of $H1^A$ and H1 on the F2 axis are much better resolved (Figure 4a) than are those of the analogous signals in the curcumin-66γCD_2ur system (Figure 4b). The broadening of the curcumin H*a*−H*e* signals of the latter system is consistent with motion of curcumin within the 66γCD_2ur complex occurring at an intermediate rate on the ^{1}H NMR time scale. The apparent absence of the H*f* signals may be due to it being sufficiently broadened as to be indistinguishable from the baseline (in contrast to the well-resolved H*f* signal seen in the presence of 66γCD_2su in Figure 4a). The broadening of the 66γCD_2ur $H1^A$ signal and the broadening of the base of the H1 multiplet is also consistent with this explanation. In contrast, the well-resolved signals of the curcumin-66γCD_2su system indicate a lesser mobility of curcumin.

An alternative explanation of the absence of the H*f* curcumin signal in the presence of 66γCD_2ur is that deuterium exchange has occurred due to keto−enol tautomerism as has been established in similar systems.[45−47] This explanation would infer that such an exchange is less effective in the presence of 66γCD_2su and the operation of a selective process.

Delivery of 66γCD_2su- and 66γCD_2ur-Bound Curcumin to a Model Membrane. The delivery of 66γCD_2su- and 66γCD_2ur-bound curcumin to a model membrane was assessed by fluorescence spectroscopy. The linked γ-CD dimers not only must provide a high level of stabilization of curcumin (Figure 2, panels c and d), but it must also demonstrate an ability to deliver and release curcumin to membranes and furthermore to the intracellular milieu. In our study, sodium dodecyl sulfate (SDS) micelles were used as a model membrane system. It is established that the fluorescence properties of curcumin are highly dependent on the polarity of the solvent/environment.[41,48,49] For instance, although curcumin is almost nonfluorescent in water, it has a measurable (2−10%) quantum yield of fluorescence (Φ_{fl}) in serum proteins, liposomes, micelles, as well as polar organic solvents.[41,48,49] This sensitivity of fluorescence of curcumin to the surrounding environment is used to demonstrate the delivery of curcumin to SDS micelles using either 66γCD_2su or 66γCD_2ur.

Figure 5 shows the UV−vis absorption and fluorescence spectra of curcumin in 66γCD_2su and 66γCD_2ur, in the absence and presence of SDS micelles in pH 7.4 phosphate buffer at 37 °C. The spectra shown in red are those of curcumin in (a) 66γCD_2su and (b) 66γCD_2ur in the absence of SDS micelles. The results show that curcumin in the linked γ-CD dimers has a negligible level of fluorescence, indicating that curcumin is in contact with water molecules while it complexes with the linked γ-CD dimers. Although curcumin is embedded in the annuli of the linked γ-CD dimers, a significant portion of the π-conjugation of curcumin is still accessible by water, which leads to efficient quenching of fluorescence. Curcumin has Φ_{fl} of 0.06 ± 0.01% and 0.04 ± 0.01% (Table 1) in the two linked γ-CD dimers. In contrast, in the presence of SDS micelles, curcumin in 66γCD_2su and 66γCD_2ur exhibits considerably higher Φ_{fl} of 2.47 ± 0.18% and 2.07 ± 0.27% (Table 1), respectively. These Φ_{fl} values indicate that 66γCD_2su- and 66γCD_2ur-bound curcumin is delivered to the SDS micelle. However, further insight can be obtained by considering the following two issues. First, is the increase of Φ_{fl} of linked γ-CD dimer-bound curcumin in the presence of micelles an indication that it is trapped in the micelle? The Φ_{fl} of curcumin captured in the micelle alone offers insight to address this issue. Curcumin has a Φ_{fl} of 2.04 ± 0.14% in the micelle alone and the similarity of this Φ_{fl} value to those of linked γ-CD dimer-bound curcumin in the presence of micelles is consistent with curcumin being trapped in the micelle. Second, the question as to whether curcumin alone or curcumin in the linked γ-CD dimers is trapped in the micelle still remains. To address this issue, we turn to a recent study that investigated the interactions between SDS and β-CD.[50] Using the hydrolysis of 4-methoxybenzenesulfonyl chloride as a probe, García-Río et al. showed that there are significant cyclodextrin-SDS interactions below the critical micelle concentration (CMC). However, the interactions between these two species are negligible above the CMC. These authors showed that the CMC and the hydrolysis kinetic parameters remain constant within experimental errors regardless of the presence of β-CD. Based on these results, García-Río et al. argued that micelles and cyclodextrins coexist independently without interactions between each other. Therefore, we inferred that the interactions between linked γ-CD dimers and SDS micelles are likely to be negligible. Although interactions between linked γ-CD dimers and SDS micelles may be absent, the Φ_{fl} results clearly show that curcumin is delivered to the micelle by the linked γ-CD dimers, which has important implications in the potential for 66γCD_2su and 66γCD_2ur to be effective delivery agents for curcumin. The mechanism of delivery is still not understood and our future work will address this issue.

CONCLUSION

We report the use of the linked γ-CD dimers, 66γCD$_2$su and 66γCD$_2$ur, to suppress the decomposition of curcumin in the aqueous environment at physiological conditions for the first time. Encapsulation using either 66γCD$_2$su or 66γCD$_2$ur leads to significant stabilization of curcumin, increasing the half-life by at least 180−750-fold. We demonstrate that the 1:1 association of curcumin with 66γCD$_2$su and 66γCD$_2$ur is cooperative, with curcumin occupying both γ-CD annuli. The cooperative binding of curcumin by 66γCD$_2$su and 66γCD$_2$ur has high stability constants of 8.7×10^6 and 2.0×10^6 M^{-1}, respectively. While 66γCD$_2$su- and 66γCD$_2$ur-bound curcumin are nonfluorescent, the significant increase of fluorescence quantum yield in the presence of surfactant micelles indicates delivery of curcumin to the micelle by 66γCD$_2$su and 66γCD$_2$ur. In short, 66γCD$_2$su and 66γCD$_2$ur show considerable promise for in vivo delivery of curcumin owing to their ability to stabilize curcumin effectively.

ASSOCIATED CONTENT

Supporting Information. UV−vis absorption spectra of curcumin in α-CD and β-CD, degradation of curcumin in 66βCD$_2$su and 66βCD$_2$ur, a table summarizing the chemical shifts of curcumin, 66γCD$_2$su, 66γCD$_2$ur, and their complexes, fluorescence quantum yield results, and molar absorptivities of curcumin, 66γCD$_2$su-curcumin, and 66γCD$_2$ur-curcumin complexes. This material is available free of charge via the Internet at http://pubs.acs.org.

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ACKNOWLEDGMENT

This work was supported in part by a research grant from the Australian Research Council (DP0878100). The authors acknowledge Dr. Philip Clements for assistance with the 2D NMR experiments.

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