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A Spectroscopic and Molecular Modelling Study of the Nature of the Association Complexes of Nile Red with Cyclodextrins

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Abstract

The polarity-sensitive fluorescent dye Nile Red forms association complexes with various cyclodextrins in aqueous solution. The formation of such association complexes has a significant effect on the Nile Red fluorescence, with the largest effect being observed in γ-cyclodextrin solution. When γ-cyclodextrin is used to increase the Nile Red concentration in solution, the absorption spectrum shows a large blue shift indicating the formation of an inclusion complex, but surprisingly the Nile Red fluorescence is strongly suppressed. A proposed explanation for this observation involves the formation of 1:2 host:guest complexes, in which the Nile Red guests are included as relatively non-fluorescent dimers. When the solutions were prepared by adding γ-cyclodextrin to a near-saturated aqueous solution of Nile Red (so that 1:1 or 2:1 complexation should be favoured), significant fluorescence enhancement was observed. Analysis of the fluorescence enhancement as a function of host concentration indicated the formation of 2:1 host:guest complexes in these solutions. However, electrospray mass spectroscopic studies show no evidence for the formation of any such inclusion complexes. Furthermore, molecular modelling shows that the formation of a complex involving full insertion of Nile Red in the γ-cyclodextrin cavity is not stable, and will quickly eject the Nile Red guest molecule. These modelling results suggest that an association complex involving capping (via the association of Nile Red parallel to the cavity opening, or by partial insertion into the cavity) of the γ-cyclodextrin cavity by one or two Nile Red molecules is much more likely.

Introduction

Nile Red (Figure 1a) is an extremely polarity-sensitive fluorescent probe molecule, being nearly nonfluorescent in aqueous solution, but highly fluorescent in less polar media [1–4]. It is also extremely hydrophobic, and is nearly insoluble in aqueous solution. The nonradiative decay of the first excited singlet state of Nile Red is known to involve a twisted intramolecular charge transfer (TICT) process [5], which is a highly polarity-sensitive process. It has been used as a polarity-sensitive fluorescent probe to determine the polarity of homogeneous binary solvent systems [6]. It has also been widely used as a fluorescent probe for the study of a variety of heterogeneous systems, including hydrophobic proteins [2, 7], protein-surfactant complexes [3, 4], zeolites [8], Langmuir-Blodgett films [9], micelles [10, 11], membranes [11], and liquid crystals [12].

Cyclodextrins (CDs) are cyclic oligomers of glycopyranose, which form truncated cone shapes with relatively nonpolar internal cavities in aqueous solutions [13, 14]. CDs form supramolecular host-guest inclusion complexes with a wide range of guest molecules in solution. In such a complex, a smaller guest molecule becomes included within the internal cavity of the CD host. There are three main CDs: α-CD, β-CD, and γ-CD (shown in Figure 1b), which contain 6, 7, and 8 glycopyranose monomers, respectively, and which thus have increasingly large cavities. These naturally-occurring, or native, CDs can also be chemically modified on the hydroxyls which line the upper and lower rims of the cavity, yielding modified CDs with improved properties [15]. An example used in this work is the set of hydroxypropylated cyclodextrins (HP-α-CD, HP-β-CD, and HP-γ-CD), in which some of the primary hydroxyl hydrogens have been replaced by $-CH_2CHOHCH_3$ groups. Due to their availability, aqueous solubility, range of cavity sizes, and ability to be modified, CDs are by far the most versatile and widely used and studied host molecules.

The host-guest inclusion complexes of CDs can be studied by a variety of techniques; one of the most sensitive and informative is fluorescence spectroscopy. This technique works especially well if the guest molecule is a highly polarity-sensitive fluorescence probe, since the effect of inclusion from aqueous solution into the relatively nonpolar CD cavity will be very large for such a guest. For example, previous work in our group used the highly polarity-sensitive

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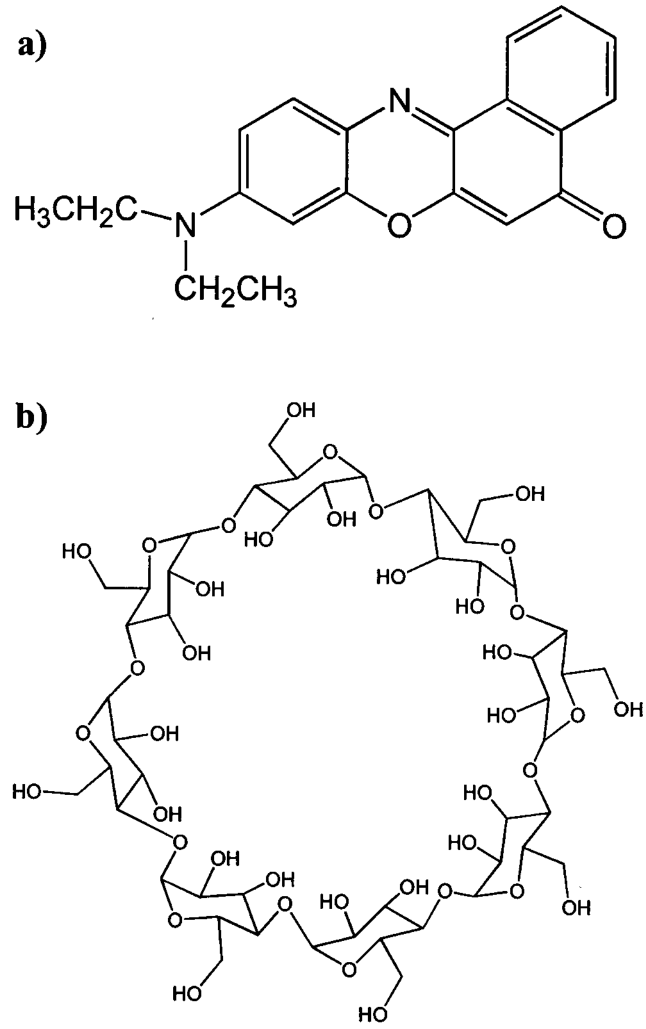

Figure 1. Structures of Nile Red and γ-Cyclodextrin.

anilinonaphthalene sulfonate (ANS) fluorescent probes as guests in the study of the host-guest inclusion complexes of native and modified CDs [16–18]. Nile Red is another ideal fluorescent probe for the study of CD inclusion complexes. Its highly hydrophobic nature should provide a large driving force for inclusion into the relatively non-polar CD cavity in aqueous solution, while its high polarity-sensitivity should result in a large changes in its fluorescence upon inclusion.

Only one previous study has reported the inclusion of Nile Red into cyclodextrins, that of Srivatsavoy [19]. In that work, UV-visible absorption as well as steady-state and time-resolved fluorescence spectroscopy and fluorescence quenching were used to study the inclusion complexes of Nile Red in β-CD and γ-CD. The author concluded that multiple types of complexes were formed with both CDs. For example, the experimental results indicated the presence of at least two emitting and one non-emitting species in the case of γ-CD. Significant differences between the two CDs were observed, with the main difference being a much longer lifetime in β-CD as compared to γ-CD. While multi-exponential decays were observed in both CDs, the major component had a lifetime of 40 ps in γ- CD, whereas the major component in β-CD had a lifetime of 1.2 ns in β-CD. This difference was interpreted to be a result of the effect of inclusion on the TICT decay process in Nile Red, with the author concluding that inclusion into β-CD has negligible effect, whereas inclusion into γ-CD was hypothesized to significantly affect the TICT decay pathway. In order to significantly reduce the Nile Red lifetime, inclusion into γ-CD would have to result in a significant enhancement of the TICT decay process in Nile Red; the author did not propose a mechanism whereby such an enhancement could result from inclusion into γ-CD. Furthermore, the author made no suggestions as to the nature of the host-guest inclusion complexes, i.e. the geometry and mode of inclusion of the Nile Red guest into the CD hosts, or even as to the stoichiometry of the host-guest complexes (1:1, 1:2, 2:1, etc.), which might explain the observed differences between the β-CD and γ-CD results.

In this paper, we use UV-visible and steady-state fluorescence spectroscopy, as well as electrospray mass ionisation spectroscopy, to experimentally study the nature of these Nile Red-CD complexes. We further use molecular modeling to test our hypotheses on these structures, and suggest the most likely geometries and stoichiometry of the association complexes. In fact, in the case of Nile Red in γ-CD, we suggest an association complex involving capping of the larger rim of the γ-CD cavity by Nile Red, as opposed to an inclusion complex involving penetration of the Nile Red guest into the γ-CD cavity.

Experimental

Nile Red, α-CD, β-CD, HP-α-CD, HP-β-CD, and HP-γ-CD were obtained from Sigma-Aldrich Chemical Co.; γ-CD was obtained from Cerestar USA. All compounds were used as received. Tests of the water content of the CDs showed values ranging from 3.3 to 11.8% for all of the CDs used (based on mass loss after heating for 4 hours in a vacuum oven at 180 °C). The CDs were not dried before use, however the calculated CD concentrations were corrected using the determined water content values.

Solutions for fluorescence studies were prepared using ultrapure water by one of two methods. In *Method A*, an excess of Nile Red and a measured amount of the CD of interest (to give [CD] = 10 mM) were added to a 50 mL volumetric flask, then filled to the mark with water. The solutions were sonicated for ten minutes, then left in the dark for 24 hours to insure equilibration. The solutions were then filtered to remove undissolved solid Nile Red. In *Method B*, an excess of Nile Red was added to a 250 mL volumetric, which was then filled with water. This solution was then magnetically stirred for two hours, then left in the dark for 24 hours. The solutions were then filtered to remove undissolved solid Nile Red, yielding a stock solution of Nile Red in the absence of CD, albeit at a very low concentration. This stock solution was then used to prepare solutions with various CDs at various concentrations by adding a measured amount of the CD to a volumetric flask, then diluting to the mark with the Nile Red solution.

Absorption spectra were measured on a Cary 50 Bio UV-Visible Spectrophotometer (UPEI). Fluorescence spectra were obtained on a Photon Technology International LS-100 luminescence spectrometer (UPEI) in 1 cm^2 quartz fluorescence cells, with an excitation wavelength of 540 nm. All spectra were obtained at 21 $\pm$ 2 °C. Solutions were not oxygen-purged, as preliminary investigations showed a negligible effect of purging on the observed fluorescence intensity (< 5%). Fluorescence enhancements (F/F_0) were determined as the ratio of the integrated area under the corrected fluorescence spectrum (I_F vs. wavenumber) of Nile Red in the presence relative to the absence of the CD of interest.

Electrospray ionisation mass spectroscopy (ESI-MS) experiments were performed on a Quattro II Micromass (U.K.) spectrometer (CEN de Saclay). Samples were injected at 10 mL/min, with a source temperature of 80 °C and capillary tension maintained at +3.35 KV. The cone voltage ranged from 10–70 V; at 20 V the skimmer voltage was 1.9 V. In a typical experimental setting, 100 μL of an ethanolic solution of Nile Red and γ-CD (1:1 or 2:1 mole ratio) was prepared and introduced through a Harward Apparatus syringe pump. The ions were detected by scanning the first quadrupole and the mass range was monitored from m/z = 80 - 2000 in 7 s. At least 50 scans were averaged to obtain representative spectra.

Molecular modeling calculations were performed on HyperChem 6Mm$^+$ (U de M) in gas phase and in solvent box (ethanol) modes. The minimisation of energy was performed using the technique described in the HyperChem instruction manual, and in reference 20. In a first phase, 1600 Polak-Ribler iterations were applied. A small incremental energy was applied in a second phase (0.001 ps for a total of 1 ps, 370 K) for selected insertion models only. All calculated complex structures (1:1, 2:1 and 1:2 stoichiometry) and energy data are available upon request from CKJ (U de M).

Results and discussion

Absorption and fluorescence spectroscopy studies

Due to the low solubility of Nile Red in aqueous solution, Method A was first used to prepare Nile Red-CD solutions, in which excess Nile Red was added to a 10 mM aqueous solution of the CD of interest, then filtered. This method takes advantage of the solubilizing abilities of the CDs to get more Nile Red into solution then would be possible in an aqueous solution without CD. However, this method has two disadvantages: there is no reference solution to enable calculation of the fluorescence enhancement of the CD, and the amount of Nile Red in solution is different for each CD. The absorption and fluorescence results for these solutions are presented in Table 1, while the fluorescence spectra are shown in Figure 2.

The maximum absorbance values listed in Table 1 show that Nile Red was most soluble in the modified β-CD solutions (Me-β-CD and HP-β-CD), followed by γ- CD, β-CD, and HP-γ-CD. The absorption maximum wavelength was similar in the three β-CDs with a value of 585 $\pm$ 2 nm, while that in the two γ-CDs was significantly blue-shifted relative to this, with values of 550 and 554 nm. These absorption results indicate that the γ-CD cavity has the largest effect on the Nile Red absorption spectrum, with the significant blue-shift observed indicating that Nile Red is experiencing a less polar environment in the case of the γ-CDs compared to the β-CDs. These absorption results suggest the formation of inclusion complexes of Nile Red into the CD cavities, with a larger degree of penetration into the cavity in the case of the γ-CDs.

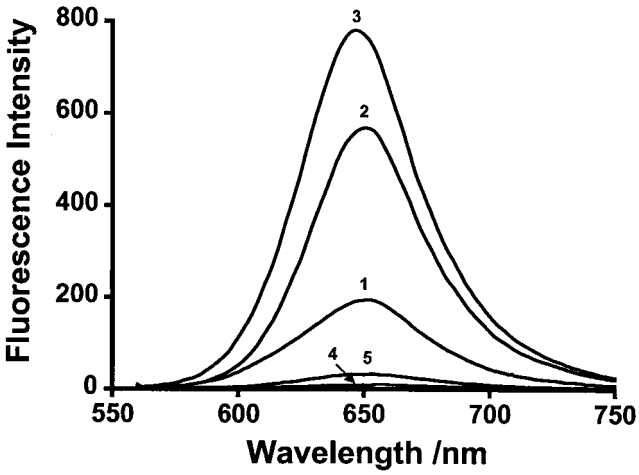

Figure 2. Fluorescence spectra of Nile Red in various 10 mM CD solutions prepared by Method A. **1** β-CD; **2** Me-β-CD; **3** HP-β-CD; **4** γ-CD; **5** HP-γ-CD.

In the case of fluorescence, however, a very interesting result is obtained, as can be seen in Figure 2: strong fluorescence is observed from Nile Red in the three β-CDs, while Nile Red in the two γ-CDs is observed to be practically non-fluorescent. Since there is no proper reference solution (i.e. a solution with no CD), the fluorescence enhancement (F/F_0) values listed in Table 1 are defined relative to Nile Red in β-CD. The fluorescence is thus seen to increase in the two modified β-CDs relative to β-CD itself, but is strongly suppressed in the two γ-CDs, and especially in the case of the parent γ-CD itself. This pattern is still maintained if these relative fluorescence ratios are corrected to take into account the different absorbances of these 5 solutions at the excitation wavelength of 540 nm, due to the differing amounts of Nile Red in each solution. Again, Nile Red in the modified β-CDs show enhanced fluorescence relative to that in β-CD, while fluorescence is significantly reduced in the two γ-CDs relative to β-CD.

This reduced fluorescence in γ-CD and HP-γ-CD is unexpected, and is contrary to the absorption results, in which the large blue-shift observed in the absorption spectrum in these two CDs relative to that in the β-CDs indicate that Nile Red is experiencing the least polar environment in the γ-CDs. Considering the polarity- sensitivity of Nile Red, its fluorescence is expected to be enhanced in a less polar environment, such as that experienced in the complex with the γ-CDs, which would seem to provide a better size match between the guest and host than in the case of the β-CD cav-

Table 1. Absorption and fluorescence parameters for the aqueous Nile Red-CD solutions prepared by *Method A*

Cyclodextrin	Absorption			Fluorescence		
	A_{540}	A_{max}	$\lambda_{A,max}$/nm	$\lambda_{F,max}$/nm	F/F_0	$F/F^*_{0,corr}$
β	0.060	0.082	583	649	1.0	1.0
Me-β	0.140	0.209	585	646	3.8	1.6
HP-β	0.105	0.159	587	650	2.6	1.6
γ	0.100	0.109	550	661	0.07	0.04
HP-γ	0.070	0.079	554	646	0.02	0.02

*Corrected for the absorbance at 540 nm.

ities. Thus, there must be other factors at work besides the polarity effect of the cavity. One possibility is that the larger γ-CD allows for the inclusion of two Nile Red molecules in a single cavity, forming a 1:2 host:guest complex, whereas the smaller β-CD cavity only allows for the formation of 1:1 complexes. Formation of such inclusion complexes involving two guest molecules in a single γ-CD cavity has been previously reported in the literature, for example in the case of pyrene as guest molecule [21]. It is possible that such a Nile Red dimer would show low fluorescence relative to that of the monomer, due to self-quenching by energy transfer. The formation of such a 1:2 complex would be encouraged by Method A, in which an excess of solid Nile Red is available to be solubilized by the CDs.

To test this idea of the formation of a relatively non-fluorescent 1:2 complex in the case of γ-CD, additional γ-CD was added to this solution. This addition of more γ-CD resulted in a smooth increase in fluorescence from this solution. This is consistent with the expected shifting of the equilibrium from 1:2 host:guest complexes to 1:1 host:guest complexes as the host:guest ratio is increased. The 1:1 γ-CD:Nile Red complexes would be expected to show enhanced fluorescence, in light of the blue shifted absorption spectra observed, and thus an increase in the concentration of 1:1 complexes would explain the observed fluorescence increase with increasing γ-CD in solution.

To further test this explanation of the reduced fluorescence in γ-CD and HP-γ-CD solution as arising from the formation of relatively non-fluorescent 1:2 complexes, the solutions were also prepared by Method B, in which a near-saturated solution of Nile Red in water was prepared in the absence of CDs. This method has the advantages of providing a reference solution with no CD to allow for calculation of the CD-induced fluorescence enhancement, as well as providing solutions containing the same amount of Nile Red. The major disadvantage is that these solutions contain a much lower concentration of Nile Red than those prepared by Method A, making absorption spectra very difficult to measure, and fluorescence spectra relatively weak as well. The absorption and fluorescence results for the solutions prepared by this method are presented in Table 2, while the fluorescence spectra are shown in Figure 3. The absorption results show that the solutions all had similar low absorbances at the excitation wavelength of 540 nm, ranging from 0.019 in the absence of CD to 0.022 in the cases of β-CD and HP-β-CD. This is a factor of 3 smaller than the lowest absorbance obtained using Method A. Unfortunately, the absorption spectra were to weak to clearly assign the absorption maxima. The fluorescence results show a very different trend than in the case of Method A: fluorescence enhancement is observed in the case of every CD, and the largest enhancement is observed in the case of γ-CD. Furthermore, the fluorescence spectrum is blue-shifted by approximately 35 nm in γ-CD as compared to all of the other CDs (including HP-γ-CD). These results indicate that Nile Red is experiencing the least polar cavity in the case of its complex with γ-CD. The observation of strong fluorescence from this γ-CD solution indicates that 1:2 complexes are not being formed; this makes sense in this case, as the guest:host ratio is exceedingly small.

Table 2. Absorption and fluorescence parameters for the aqueous Nile Red-CD solutions prepared by *Method B*

Cyclodextrin	A_{540}	$\lambda_{F,max}$/nm	F/F_0
None	0.019	656	1.0
β	0.022	650	5.3
Me-β	0.022	646	7.0
HP-β	0.020	648	6.4
γ	0.021	615	9.6
HP-γ	0.021	649	3.1

The stoichiometry of the γ-CD:Nile Red complexes, as well as the association constant(s) involved in the complex formation process, can be determined by looking at the fluorescence enhancement as a function of γ-CD concentration, as shown in Figure 4. If only 1:1 complexes form, then the enhancement is related to the added host concentration ($[CD]_0$) according to the following equation [22, 23]:

$$F/F_0 = 1 + (F_\infty/F_0 - 1)\frac{[CD]_0\,K}{1 + [CD]_0\,K} \qquad (1)$$

where F_∞/F_0 is the fluorescence enhancement when all of the guests have been included (i.e. at infinite host concentration). Furthermore, the double-reciprocal plot (or Benesi-Hildebrand plot [24]) of $1/(F/F_0 - 1)$ *versus* 1/[CD] will be linear if only 1:1 complexes form, but will be non-linear if higher-order complexes form [22]. The double-reciprocal plot is also shown in Figure 4; it is clearly non-linear. Fur-

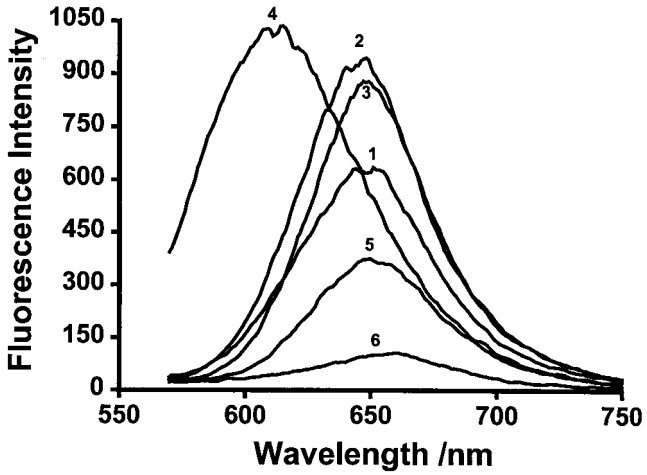

Figure 3. Fluorescence spectra of Nile Red in various 10 mM CD solutions prepared by Method B. **1** β-CD; **2** Me-β-CD; **3** HP-β-CD; **4** γ-CD; **5** HP-γ-CD; **6** no CD.

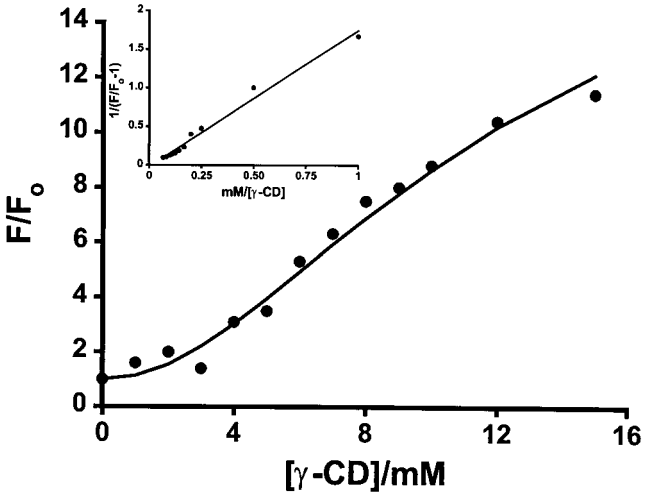

Figure 4. Fluorescence enhancement (F/F_0) of Nile Red as a function of γ-CD concentration in solutions prepared by Method B. The solid line shows the best fit line to Equation 2 for 2:1 complexation. The inset shows the double-reciprocal plot of $1/(F/F_0 - 1)$ *versus* $1/[\gamma\text{-CD}]$.

thermore, Equation (1) did not provide a good fit to the experimental data. Thus, higher-order complexes are clearly involved. If 2:1 host:guest complexes form from the complexation of an initially-formed 1:1 complex by a second host, then the measured enhancement will be related to the host concentration according to the following equation [25]:

$$F/F_0 = \frac{1 + F_1/F_0\ K_1\ [CD]_0 + F_2/F_0\ K_1\ K_2\ [CD]_0^2}{(1 + K_1\ [CD]_0 + K_1\ K_2\ [CD]_0^2)} \quad (2)$$

where F_1/F_0 and F_2/F_0 are the fluorescence of the 1:1 and 2:1 complexes, respectively, relative to free Nile Red, K_1 is the equilibrium constant for 1:1 complex formation, and K_2 is the equilibrium constant for the addition of a second host to a 1:1 complex to form a 2:1 complex. The solid line in Figure 4 shows the line of best fit of the experimental data to Equation (2); this equation clearly provides an excellent fit. The equilibrium constants obtained were: $K_1 = 30 \pm 3\ M^{-1}$ and $K_2 = 272 \pm 18\ M^{-1}$. Thus, in the case of γ-CD:Nile Red complexes prepared by Method B, 2:1 host:guest complexes dominate; this is consistent with the very high host:guest ratio in these solutions.

In summary, the absorption and fluorescence results indicate the formation of inclusion complexes of Nile Red in the various CDs studied. In the case of γ-CD, Nile Red solutions prepared by Method A show extremely low fluorescence, which we propose to be the result of the formation of non-fluorescent 1:2 complexes, in which the included Nile Red dimers undergo self-quenching. On the other hand, in the case of the analogous solutions prepared by Method B, in which the host:guest ratio is much higher, highly fluorescent 2:1 γ-CD:Nile Red complexes are formed, with significant enhancement of the Nile Red fluorescence compared to free Nile Red in solution. However, these absorption and fluorescence spectroscopic studies provide only indirect evidence for inclusion. Furthermore, although they do determine the stoichiometry of the complexes formed (1:1, 1:2 and 2:1), and the observed fluorescence enhancements and absorption and fluorescence blue shifts in the case of the γ-CD:Nile Red complexes suggest inclusion of the Nile Red into the relatively non-polar CD cavity, they provide no information on the exact nature of these complexes, such as the relative host:guest geometry and orientation. Thus, further experimental studies of these complexes were performed using electrospray ionisation mass spectroscopy (ESI-MS), and calculations using Molecular Modelling.

Electrospray ionisation mass spectroscopy (ESI-MS) studies

ESI-MS spectra were measured to confirm the presence of γ-CD-Nile Red complexes in solution, and to establish the ratio of dye to host molecule in the major species observed. Spectra of the Nile Red and γ-CD substrates were obtained without difficulty in ethanol as solvent, especially in positive ion (PI) mode. Figure 5a shows the ESI-MS PI spectrum of Nile Red, which produced an m/z 319 protonated ion. Figure 5b shows the ESI-MS PI spectrum of γ-CD, which produced the expected m/z 1297 protonated ion. With the application of higher cone voltage, successive losses (up to six) of glucose units were observed (MH^+-n162). Thus, the purity of both substrates was established by ESI-MS, allowing the supramolecular host-guest inclusion complexes of these two substrates to be considered next.

However, there was no evidence of any supramolecular complexes of Nile Red in γ-CD; no signals corresponding to either the molecular mass of any expected adducts, nor to any poly-charged ions of such adducts, were observed. This was in spite of the use of a wide range of cone voltage (10–70 V) and various modifications to the solvent system, including the use of H_2O, D_2O, methanol, ethanol, trifluoroethanol, acetonitrile, and mixtures of these solvents. When any solution expected to contain γ-CD-Nile Red complexes was prepared, by either direct addition of the two substrates, or addition of an excess of Nile Red followed by filtration to remove undissolved particles, a striking absence of ions of high masses which could be assigned to 1:1, 2:1 or 1:2 complexes was observed. Figure 6 shows typical ESI-MS spectra of a γ-CD-Nile Red mixture, at two different

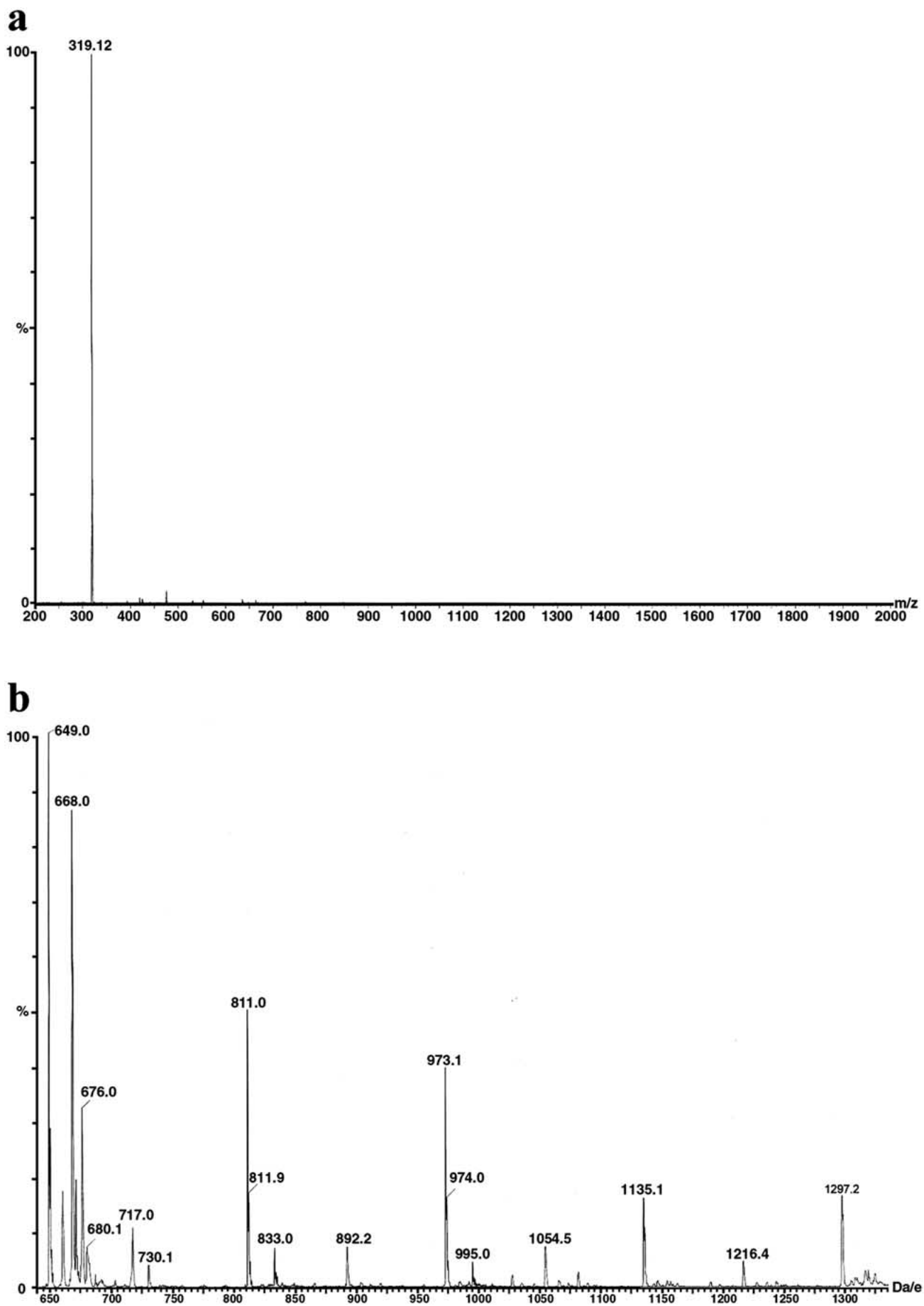

Figure 5. ESI-PI mass spectrum of (a) Nile Red (1% solution in ethanol) and (b) γ-CD using Micro Mass Quattro II.

scan energies. There are two higher mass ions observable in Figure 6a (lower scan energy), although with very weak intensity, at m/z 1729 and 1949. Of these, only the first at 1729 is assignable to any complex: 1729 = 1:1 complex + SO_4^{2-} + H_2O. However, when fresh solutions were prepared in ethanol, both of these ions were absent. Furthermore the intensity of the 1729 peak decreased at higher scan energy, as can be seen in Figure 6b.

Thus, despite the evidence of inclusion of Nile Red into γ-CD in water obtained from the absorption and fluorescence studies described in Section 3.1, the expected inclusion complexes were not detected by ESI-MS in ethanol, water, or any other solvent. We must conclude that although ESI-MS has been shown in the literature to be a very sensitive technique for the detection of other host-guest inclusion complexes [20, 26–32] (as have other MS techniques, such as MS-FAB [33]), it is not appropriate for detection of these γ-CD-Nile Red complexes. Several reasons can be proposed for this. The first is the presence of large amounts of uncomplexed γ-CD which could affect the sensitivity of the measurement [30]. Another reason is the possibility that these complexes cannot tolerate a cone voltage even as low as 10 V, and dissociate into free host and guest molecules. A third possible explanation could of course be that there is a lack of the significant, stable insertion of Nile Red into the γ-CD cavity originally predicted based on the fluorescence results described in Section 3.1. In order to further study this possibility, molecular modelling calculations were performed to investigate the possible interactions between Nile Red and γ-CD in solution.

Molecular modelling studies

In order to help explain the absence of signals from inclusion complexes in the ESI-MS experiments, we examined the host and guest compounds, and the complexes formed between them, using molecular modelling (MM). MM has been successfully applied to a wide variety of host-guest inclusion complexes of cyclodextrins [34–40]. Modelling of the separate host and guest showed that the inner cavity of the γ-CD structure is full of hydrogen bonds, while the Nile Red can be best described as a dipolar structure (resonance form with a charged diethylamino cation on one side and a conjugated α,β-unsaturated anion on the opposite side). The host and guest were then combined together using complete or partial insertion of the Nile Red into the γ-CD cavity. Calculations were performed for the nine different structures listed and described in Table 3, which result from a consideration of the different ways a 1:1 complex could form between γ-CD and Nile Red. First of all, inclusion could occur into the upper or lower rim of γ-CD. Since Nile Red has an elongated shape, its inclusion could occur axially (inclusion parallel to its long axis) or equatorially (inclusion parallel to its short axis). Due to the lack of symmetry in the molecule, there are two different directions for the Nile Red in each of these types of inclusion: for axial inclusion, either the end with the diethyl amine group or that with the exposed aromatic ring could enter the cavity (structures **6** and **8**); while in equatorial inclusion, the side with the oxygen atoms could be pointing towards (structures **1** and **3**) or away (structures **2** and **4**) from the cavity. In the case of axial inclusion, the structures with the dimethylamino group entering the cavity were not considered, since this group is very bulky. This gives six structures. In addition, it is possible to imagine complexes with no actual inclusion, but in which the Nile Red is complexed flat against the γ-CD cavity opening, thus serving as a cap; calculations were performed for the structure with Nile Red capping the lower rim (structure **5**). In the first six structures, Nile Red was only partially inserted into the cavity at the beginning of the calculation. Two additional structures were thus considered: the axial inclusion complexes in which the Nile Red was forced inside the γ-CD cavity at the start of the calculation (structures **7** and **9**).

Table 3 also shows the association energies calculated for these nine structures. As can be seen, there is not a large energetic difference between these structures. The most stable structures were the axial inclusion complexes in which the Nile Red was forced inside the γ-CD cavity, and in particular structure **7** involving axial inclusion into the upper (larger) larger cavity opening (Type II inclusion). However, an interesting phenomenon took place with these two structures: as the calculation proceeded, the Nile Red eventually left the cavity, it did not stay inside. This was also observed if small thermal shots of energy were added in order to do a Molecular Dynamics simulation, and in solvent (ethanol) box calculations. Thus, if the Nile Red is forced inside the cavity, then these calculations show that it will soon exit, and that the most stable structures involve at most only capping or partial insertion of Nile Red into the γ-CD cavity.

Calculations were also performed on 1:2 and 2:1 γ-CD:Nile Red structures, again using various orientations of the guests and hosts. In all of these cases, much higher energies were obtained than in the case of 1:1 complexes. For 2:1 complexes, inclusion was assumed to be axial (to allow two γ-CD molecules to cap the two ends of a single Nile Red), and the most stable structure was that in which one γ-CD capped the diethylamino end of Nile Red by its upper rim, while the other γ-CD capped the other end of Nile Red by its lower rim (145.9 kcal mol^{-1}). For 1:2 complexation, the most stable structure involved one Nile Red axially included into the upper γ-CD rim by its dimethylamino end, while the second Nile Red was partially included into the lower rim by its aromatic end (104.0 kcal mol^{-1}).

The conclusion from these modelling studies is that the inclusion of one (or of course two) Nile Red molecules into the γ-CD cavity is difficult at best, and perhaps impossible. Specifically, under the time-frame of mass spectroscopy measurements, the complexes with fully inserted Nile Red cannot be observed, since the γ-CD seems to push the Nile Red out of the cavity to a large extent. The results also seem to indicate that a complex in which the polar Nile Red molecule is capping the γ-CD cavity, as opposed to a true full inclusion complex, is a strong possibility for these structures. The capping could occur either with the Nile Red flat against the cavity rim, or by partial insertion into the cavity, giving a *partial insertion* inclusion complex.

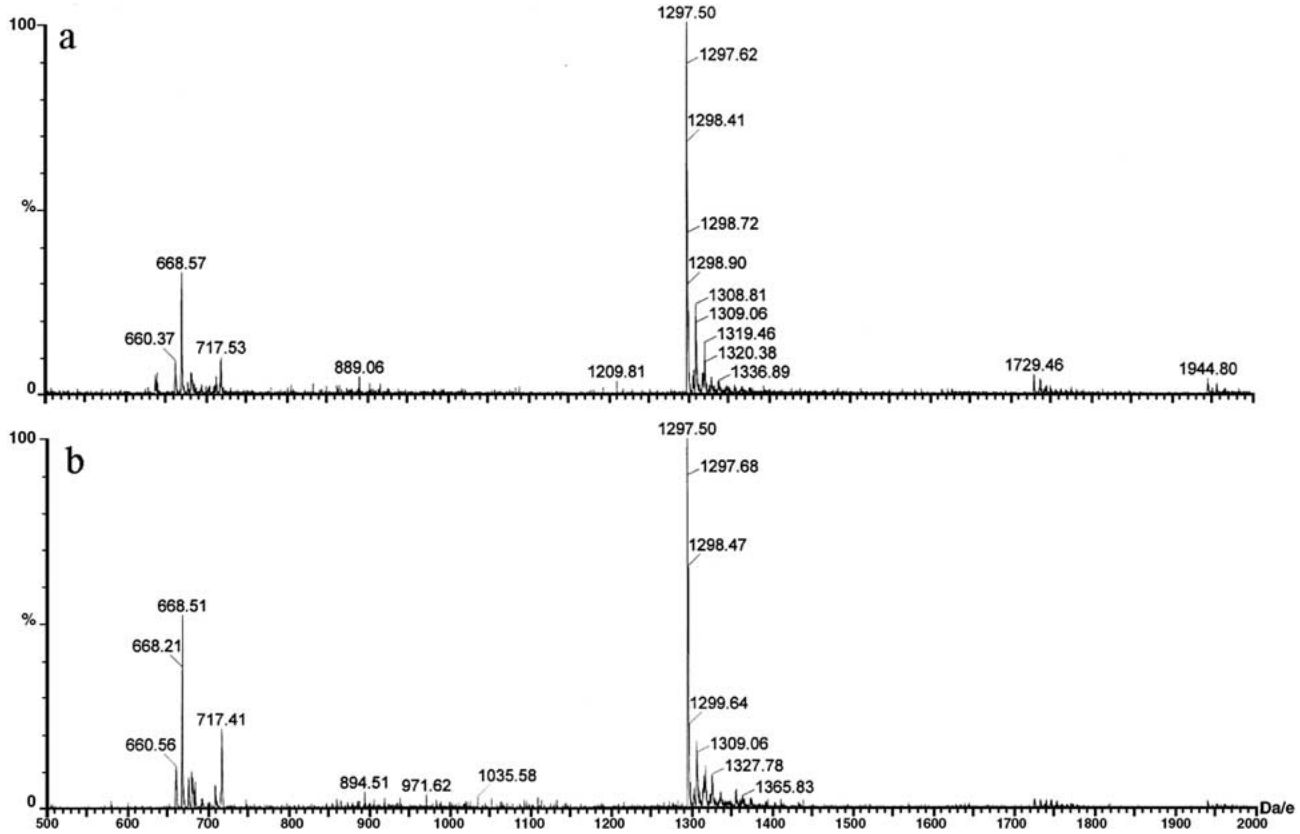

Figure 6. ESI-PI mass spectrum of γ-CD (1% solution in ethanol) using Micro Mass Quattro II.

Table 3. 1:1 γ-CD-Nile Red structures investigated using molecular modeling

Structure	Rim of γ-CD	Type of Inclusion	Nile Red Direction	Energy (kCal mol^{-1})
1	Upper	Equatorial	Oxygens toward cavity	96.739
2	Upper	Equatorial	Oxygens away from cavity	99.986
3	Lower	Equatorial	Oxygens toward cavity	96.944
4	Lower	Equatorial	Oxygens away from cavity	96.616
5	Lower	Capping	Parallel with γ-CD cavity rim	96.512
6	Upper	Axial	Diethylamine end towards cavity	95.145
7	Upper	Axial	Diethylamine end towards cavity (forced inside at start)	90.781
8	Upper	Axial	Carbonyl end towards cavity	94.863
9	Upper	Axial	Carbonyl end towards cavity (forced inside at start)	92.632

There is very little difference in calculated energy to distinguish such structures. In addition, such capped adducts have been previously observed for permethylated-β-CD [36, 38]. Furthermore, the significant effect of γ-CD on Nile Red fluorescence described in Section 3.1 could still be explained based on capped or partial insertion 1:1 and 1:2 association complexes. It is clear that such complexes would be much less likely to retain their integrity under ESI-MS conditions, thus explaining our failure to observe γ-CD Nile Red complexes using this technique.

Conclusions

Fluorescence studies of Nile Red in the presence of cyclodextrins clearly show a large effect of cyclodextrins on the fluorescence properties of Nile Red in aqueous solution. A particularly interesting result is obtained for Nile Red in the presence of γ-CD. In the case in which γ-CD is used to help solubilize Nile Red, a large blue shift is observed in the Nile Red absorption spectrum, indicating that the Nile Red is experiencing a less polar cavity (suggesting inclusion into the γ-CD cavity), but the fluorescence is extremely reduced. This was proposed to be the result of the formation of 1:2 inclusion complexes, in which a relatively non-fluorescent Nile Red dimer is included in the cavity. At

higher γ-CD concentration, or at lower Nile Red concentration, a very large enhancement and blue-shift of the Nile Red fluorescence is observed, presumably due to the preferential formation of 1:1 (or even 2:1) complexes under these conditions.

The detection of these γ-CD:Nile Red host-guest inclusion complexes by ESI-MS was not possible for this system. The failure to clearly identify the 1:1 or 1:2 γ-CD-Nile Red complexes is considered to be a result of the weak formation constants for these complexes, and their inability to preserve the noncovalent host-guest binding during the ionisation/droplet evaporation process. A further reason could also be the higher concentration of the host γ-CD required for significant complex formation. It is also possible that other unexpected complexes form, such as 2:1 or 2:2, which would have masses above the m/e 2000 range investigated in this study.

Molecular modelling indicated that a true inclusion complex, in which the Nile Red is fully inserted into the γ-CD cavity, although stable is not likely for this system, and the results suggested that the complexes involved in the observed fluorescence enhancement and blue-shifting most likely involve capping the γ-CD cavity by the Nile Red, or at the most partial insertion into the γ-CD cavity. In these calculations, under the conditions of the modelling, higher-order 1:2 or 2:1 complexes were found to be of much higher energy than the 1:1 complexes. This conclusion that Nile Red forms capped complexes with γ-CD as opposed to true inclusion complexes is consistent with the inability to observe these complexes via ESI-MS; such capped complexes would be expected to be much less robust than fully inserted complexes. Furthermore, the capped complexes would still result in a large change in the Nile Red environment as compared to free dye in aqueous solution, so the observed effects of γ-CD on Nile Red fluorescence are also fully consistent with the formation of capped 1:1 and 1:2 association complexes.

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References

1. P. Greenspan and S.D. Fowler: *J. Lipid Res*. **26**, 781 (1985).
2. D.L. Sackett and J. Wolff: *Anal. Biochem*. **167**, 228 (1987).
3. J.-R. Daban, M. Samsó, and S. Bartolomé: *Anal. Biochem*. **199**, 162 (1991).
4. J.-R. Daban, M. Samsó, and S. Bartolomé: *Anal. Biochem*. **199**, 169 (1991).
5. A.K. Dutta, K. Kamada, and K. Ohta: *J. Photochem. Photobiol. A:Chem*. **93**, 57 (1996).
6. P.K. Kipkemboi and A.J. Easteal: *Aust. J. Chem*. **47**, 1771 (1994).
7. D.L. Sackett, J.R. Knutson, and J. Wolff: *J. Biol. Chem*. **265**, 14899 (1990).
8. N. Sarker, K. Das, D.N. Nath, and K. Bhattacharyya: *Langmuir* **10**, 326 (1994).
9. A.K. Dutta, K. Kamada, and K. Ohta: *Chem. Phys. Lett*. **258**, 369 (1996).
10. A. Datta, D. Mandal, S.K. Pal, and K. Bhattacharyya: *J. Phys. Chem. B* **101**, 10221 (1997).
11. M.M.G. Krishna: *J. Phys. Chem. A* **103**, 3589 (1999).
12. M. Choi, D. Jin, H. Kim, T.J. Kang, S.C. Jeoung, and D. Kim: *J. Phys. Chem. B* **101**, 8092 (1997).
13. W. Saenger: *Angew. Chem. Int. Ed. Engl*. **19**, 344 (1980).
14. J. Szelti: *Chem. Rev*. **98**, 1743 (1998).
15. A.R. Kahn, P. Forgo, K.J. Stine, and V.T. D'Souza: *Chem. Rev*. **98**, 1977 (1998).
16. B.D. Wagner and P.J. MacDonald: *J. Photochem. Photobiol. A:Chem*. **114**, 151 (1998).
17. B.D. Wagner, P.J. MacDonald, and M. Wagner: *J. Chem. Ed*. **77**, 178 (2000).
18. B.D. Wagner and S.J. Fitzpatrick: *J. Incl. Phenom. Macro. Chem*. **38**, 467 (2000).
19. V.J.P. Srivatsavoy: *J. Lumin*. **82**, 17 (1999).
20. C.K. Jankowski and J.B. Chiasson: *Spectroscopy*, submitted (2002).
21. N. Kobayashi, R. Saito, H. Hino, Y. Hino, A. Ueno and T. Osa: *J. Chem. Soc., Perkin Trans. II*, 1031 (1983).
22. A. Muñoz de la Peña, F. Salinas, M.J. Gómez, M.I. Acedo, and M. Sánchez Peña: *J. Incl. Phen. Mol. Rec. Chem*. **15**, 131 (1993)
23. C.N. Sanramé, R.H. de Rossi, and G.A. Argüello: *J. Phys. Chem*. **100**, 8151 (1996).
24. H.A. Benesi and H. Hildebrand: *J. Am. Chem. Soc*. **71**, 2703 (1949).
25. S. Nigam and G. Durocher: *J. Phys. Chem.* **100**, 7135 (1996).
26. O. Baudoin, F. Gonnet, M.P. Teulade-Fichou, J.P. Vigneron, J.C. Tabet and J.M. Lehn: *Chem. Eur. J*. **5**, 2762 (1999).
27. A. Jaus and M. Oehme: *J. Chromatogr. A* **905**, 59 (2001).
28. J. Szejtli and T. Osa: In J.-M. Lehn (ed.), *Comprehensive Supramolecular Chemistry*, Elsevier Science (Pergamon), N.Y. (2000). See also bibliography of this volume on encapsulating agents.
29. R.J. Storer: *Neurotransmissions (Sigma)* **15**, 16 (1999).
30. R. Kobetić, B.S. Jursic, S. Bonnette, J.S.-C. Tsai and S.J. Salvatore: *Tetrahed. Lett*. **42**, 6077 (2001).
31. L. Caron, S. Tilloy, E. Monflier, J.-M. Wieruszeski, G. Lippens, D. Landy, S. Fourmentin, and G. Surpateanu: *J. Incl. Phenom. Macro. Chem*. **38**, 361 (2000).
32. P. Cescutti, D. Garazzo and R. Rizzo: *Carbohyd. Res*. **302**, 1 (1997).
33. F. Caccia, R. Dispenza, G. Fronza, C. Fuganti, L. Malpezzi, and A. Mele: *J. Agric. Food Chem*. **46**, 1500 (1998).
34. K.B. Lipkowitz: *Chem. Rev*. **98**, 1829 (1998).
35. G. Fronza, A. Mele, E. Redenti and P. Ventura: *J. Org. Chem*. **61**, 909 (1996).
36. J. Ramirez, F. He, and C.B. Lebrilla: *J. Am. Chem. Soc*. **120**, 7387 (1998).
37. E. Estrada, I. Perdomo-López, and J.J. Torres-Labandeira: *J. Org. Chem*. **65**, 8510 (2000).
38. G. Grigorean and C.B. Lebrilla: *Anal. Chem*. **73**, 1684 (2001).
39. M. Oana, A. Tintaru, D. Graviliu, O. Maior and M. Hillebrand: *J. Phys. Chem. B* **106**, 257 (2002).
40. I. Pastor, A. Di Marino and F. Mendicuttu: *J. Phys. Chem. B* **106**, 1995 (2002).