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Fluorescence Suppression of 7-Methoxycoumarin upon Inclusion into Cyclodextrins

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Abstract

The fluorescence intensity of 7-methoxycoumarin (7MC) in aqueous solution is found to significantly decrease upon addition of various cyclodextrins. This observed phenomenon is described as *fluorescence suppression*, to distinguish it from fluorescence reduction via bimolecular quenching. The decrease in fluorescence of 7MC is proposed to be the result of the formation of a host–guest inclusion complex with cyclodextrin. Since 7MC is a polarity-sensitive fluorophore, which is less fluorescent in a nonpolar environment, its fluorescence decreases upon inclusion into the relatively nonpolar internal cavity of the cyclodextrin. The same equation used for extracting the association constant in the case of 1 : 1 host–guest inclusion-induced fluorescence enhancement is shown to be applicable to the case of fluorescence suppression. In the case of β-cyclodextrin, the degree of fluorescence suppression, as well as the value of the binding constant for formation of the inclusion complex, are found to be unaffected by modification of the cyclodextrin rims, suggesting that the molecule is completely included within the β-cyclodextrin cavity. In the case of γ-cyclodextrin, the degree of fluorescence suppression, but not the value of the binding constant, is found to be significantly affected by modification of the cyclodextrin rims, suggesting that the molecule is experiencing a less polar environment in the modified γ-cyclodextrin cavity. The binding constant is three times larger in β- as compared to γ-cyclodextrin, indicating a much better size match in the smaller β-cyclodextrin cavity.

Introduction

Cyclodextrins are cyclic amylose oligomers consisting of 6 (α), 7 (β), or 8 (γ) sugar units, with an overall truncated cone shape [1]. These cyclodextrin molecules have an internal cavity, accessible to other molecules by openings of 5.7, 7.8, and 9.5 Å for α, β, and γ, respectively [1]. A wide range of organic molecules have been shown to become included inside the cyclodextrin cavity in solution. The resulting supramolecular structure is referred to as a host–guest inclusion complex. Fluorescence spectroscopy is an extremely useful tool for studying host–guest inclusion complexes. In order to use this technique, either the host or the guest must be a fluorescent species whose fluorescence changes upon formation of the inclusion complex. Most commonly, the guest is a polarity-sensitive fluorescent probe. Since the host–guest complexation is typically carried out in aqueous solution, the internal cavity of the cyclodextrin provides a relatively nonpolar environment for the probe as compared with that experienced by the free probe. This change in local polarity upon complexation results in significant, easily measurable changes in the guest fluorescence, which allows for the study of the complexation process, including determination of the complexation constant [2]. In the majority of cases, polarity sensitive probes are more fluorescent in a nonpolar medium, so that inclusion in aqueous solution results in an enhancement of the observed fluorescence intensity. In a few cases, however, the polarity sensitive probe is actually more fluorescent in a polar medium, so that inclusion into a cyclodextrin in aqueous solution would result in a decrease in fluorescence intensity. In this paper, we report the fluorescence of just such a probe, 7-methoxycoumarin (7MC, shown in Figure 1), upon inclusion into various cyclodextrins. This interesting fluorescent probe shows solvent-dependent emission, with increasing fluorescence in solvents of increasing polarity [3], and thus is expected to show *decreased* fluorescence upon inclusion.

In addition to the parent, or unmodified, cyclodextrins α-CD, β-CD, and γ-CD, the effect of commercially available hydroxypropylated derivatives of each of these cyclodextrins will also be studied; these are referred to as HP-α-CD, HP-β-CD (shown in Figure 1), and HP-γ-CD. In these cyclodextrins, some of the primary and secondary hydroxyl groups on the upper and lower rims of the cavity have been replaced by 2-hydroxypropyl groups. This modification significantly increases the solubility of the CDs and also provides an extended nonpolar cavity.

Compared to the extensive number of reports of fluorescence enhancement of guest molecules [4], there have been relatively few reports of decreased fluorescence of a guest upon cyclodextrin inclusion in aqueous solution;

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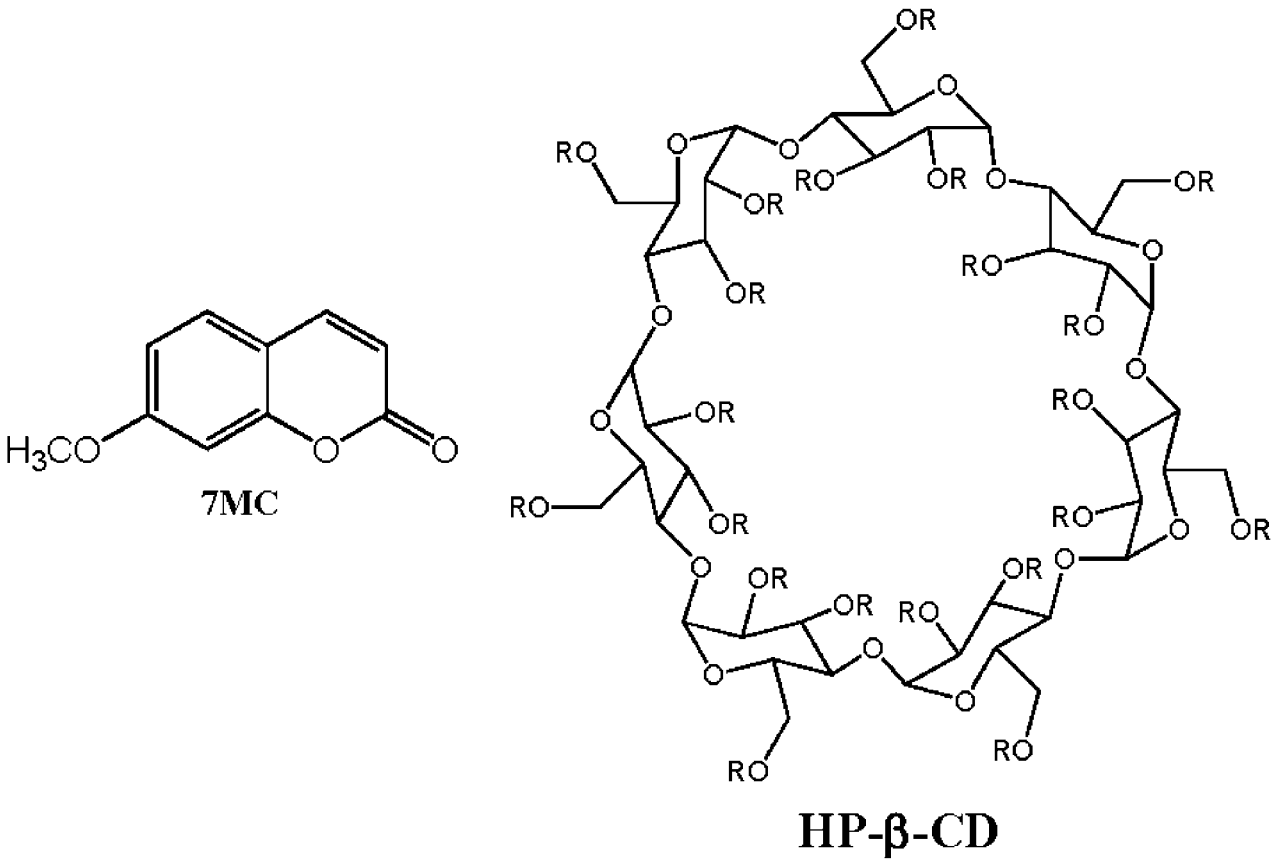

Figure 1. The structures of 7MC and HP-β-CD (R = H or $CH_2CHOHCH_2$).

such guests include acridine [5], 2-acetylnaphthalene [6], lumichrome [7], xanthone [8, 9], tryptophan [10] and 3-carboxyphenoxathiin [11]. In these previous reports, with the exception of Reference [7], the effect of inclusion was measured as an absolute decrease in fluorescence intensity in the presence and absence of cyclodextrin. In this work, we measure the effect of inclusion as a ratio of the integrated intensities in the presence and absence of cyclodextrin, and show that the equation developed in the literature to derive the complexation constant in the case of inclusion-induced fluorescence enhancement also works for the case of fluorescence suppression. This approach has a number of advantages, which will be described herein. Furthermore, whereas the previous reports have used the term *fluorescence quenching* to describe the decrease in fluorescence intensity in the presence of cyclodextrins, we suggest that the term *fluorescence suppression* is a better description of this phenomenon, for a number of reasons which we will also present herein.

Experimental

7-MC, α-CD, β-CD, HP-α-CD, HP-β-CD, and HP-γ-CD were obtained from Aldrich Chemical Co.; γ-CD was obtained from Cerestar USA. All compounds were used as received. In the case of the HP-substituted CDs, various degrees of substitution are available; in this work, those with the highest degree of substitution in each case were used. Tests of the water content of the cyclodextrins showed values ranging from 3.3 to 11.8% for all of the cyclodextrins used (based on mass loss after heating for 4 hours in a vacuum oven at 180 °C). The cyclodextrins were not dried before use, however the calculated cyclodextrin concentrations were corrected using the determined water content values.

Solutions used were 3.0×10^{-5} M in 7-MC, giving an absorbance of 0.28 at the excitation wavelength of 320 nm; this absorbance was not significantly changed even at the highest CD concentrations used.

Fluorescence spectra were obtained on a Perkin-Elmer LS-5 luminescence spectrometer, with excitation and emission monochrometer bandpasses set at 5 nm and 3 nm, respectively, or a Photon Technology International LS-100 luminescence spectrometer, with excitation and emission monochrometer bandpasses both set at 2 nm, in 1×1 cm^2 quartz fluorescence cells. 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 suppressions (F/F_o) were determined as the ratio of the integrated area under the corrected fluorescence spectrum of I_F *vs.* wavenumber (obtained using analysis software written in our laboratory) of the probe in the presence and absence of the CD of interest.

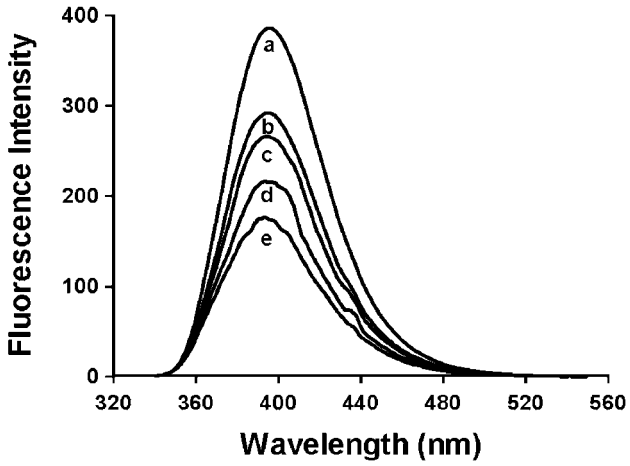

Figure 2. The fluorescence spectrum of 7-methoxycoumarin in hydroxypropyl-β-cyclodextrin solutions of various concentrations: (a) 0 M; (b) 4.8×10^{-3} M; (c) 9.7×10^{-3} M; (d) 2.4×10^{-2} M; (e) 4.8×10^{-2} M.

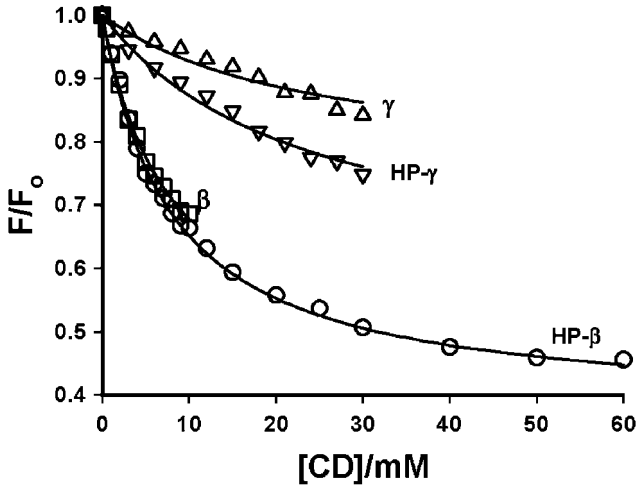

Figure 3. The effect of cyclodextrin concentration on the relative fluorescence (F/F_o) of 7-methoxycoumarin for various cyclodextrins. Experimental data: □, β-CD; ○, HP-β-CD; △, γ-CD; ▽, HP-γ-CD; ——, fit to Equation (4) (fit values given in text).

Results

Fluorescence suppression

Figure 2 shows the fluorescence emission spectrum of 7MC in water, with various concentrations of HP-β-CD added. As can be clearly seen, the intensity of the emission of 7MC decreases significantly in the presence of HP-β-CD; we refer to this as *fluorescence suppression*. This phenomenon was quantified by taking the ratio of the integrated fluorescence spectrum in the presence (F) and absence (F_o) of the CD of interest. The fluorescence suppression is then given by F/F_o. For example, in the presence of 10 mM HP-β-CD, $F/F_o = 0.66$. There was no significant spectral shift associated with the observed suppression; the maximum of the emission spectrum remained constant at 395 nm. Fluorescence suppression was also observed in the presence of other CDs. With [CD] = 10 mM; the following values of F/F_o were observed: β-CD: 0.69; γ-CD: 0.95; HP-γ-CD: 0.89. No significant fluorescence suppression of 7MC was observed in the presence of α or HP-α-CD.

Association constants for the 7MC : CD complexes

The observed values of F/F_o measured as a function of CD concentration can be used to determine the value of the association constant K, for formation of the host–guest inclusion complex. For a 1 : 1 CD : 7MC complex, K can be defined as follows:

$$\mathrm{CD} + 7\mathrm{MC} \rightleftharpoons \mathrm{CD}:7\mathrm{MC} \tag{1}$$

$$K = \frac{[\mathrm{CD}:7\mathrm{MC}]}{[7\mathrm{MC}][\mathrm{CD}]}. \tag{2}$$

In the case of fluorescence enhancement of a probe by CD inclusion, the following equation has been developed for the observed fluorescence as a function of added cyclodextrin concentration ($[\mathrm{CD}]_o$) [2, 12]:

$$F/F_o = 1 + (F_\infty/F_o - 1)\frac{K[\mathrm{CD}]_o}{1 + K[\mathrm{CD}]_o}, \tag{3}$$

where F is the integrated fluorescence intensity in the presence of CD ($\int I(\nu)d\nu$), F_o is the integrated fluorescence intensity in the absence of CD, and F_∞ is the integrated fluorescence intensity when all of the probe molecules have been complexed by CD molecules. This equation assumes that only a 1 : 1 complex is formed.

A consideration of the derivation of Equation (3) given in Reference [2] indicates that it should apply equally well for the case of fluorescence suppression, such as that observed for 7MC in CDs. The only difference is that in the case of enhancement, $F_\infty/F_o > 1$, so that F/F_o is also greater than 1 for $[\mathrm{CD}]_o > 0$, whereas in the case of suppression, $F_\infty/F_o < 1$, so that $(F_\infty/F_o - 1)$ is negative, and F/F_o is also less than 1 for $[\mathrm{CD}]_o > 0$. Figure 3 shows the plots of F/F_o *vs.* [CD] for β-CD, HP-β-CD, γ-CD, and HP-γ-CD. As can be seen from this figure, the best results are obtained with HP-β-CD, with an initial sharp decrease in F/F_o followed by a clearly-defined plateau region. This data fit extremely well to Equation (3) (using a non-linear least-squares fitting routine), as indicated by the solid line through the experimental points in Figure 3. The values obtained for the HP-β-CD : 7MC inclusion complex are $K = 120 \pm 20\ \mathrm{M}^{-1}$ and $F_\infty/F_o = 0.37 \pm 0.03$, based on the average of three experimental trials.

Equation (3) assumes that only 1 : 1 complexes are formed; this assumption can be tested using a double-reciprocal, or Benesi–Hildebrand plot [13] of $1/(F/F_o - 1)$ *versus* 1/[CD]; such a plot will be non-linear if higher-order complexes are being formed. Figure 4 shows the double-reciprocal plot for 7MC with HP-β-CD. This plot is linear, with a correlation $r = 0.997$, indicating that only 1 : 1 complexes are indeed being formed.

Good fits to Equation (3) were also obtained in the cases of β-CD, γ-CD and HP-γ-CD, with the fit values of K and F_∞/F_o listed in Table 1. In the case of β-CD, although

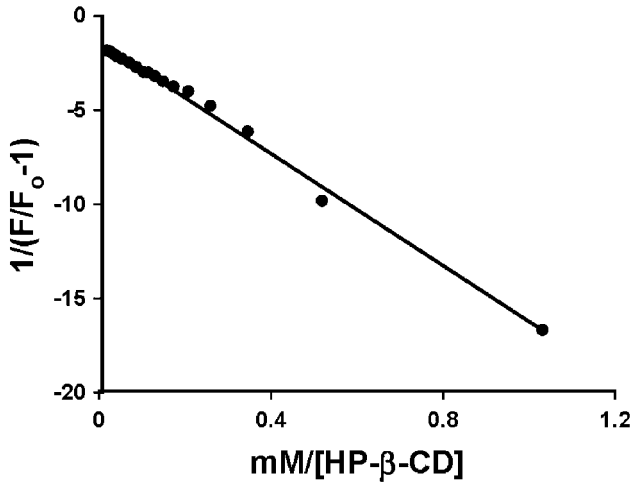

Figure 4. The double-reciprocal plot of $1/(F/F_o - 1)$ *vs.* 1/[HP-β-CD] for the inclusion of 7-methoxycoumarin in hydroxypropyl-β-cyclodextrin.

Table 1. Association constants K and maximum fluorescence suppression F_∞/F_o obtained from the fit of the experimental fluorescence data to Equation (3)

Cyclodextrin	K (M^{-1})	F_∞/F_o
β-CD	128 ± 32	0.41 ± 0.06
HP-β-CD	120 ± 20	0.37 ± 0.03
γ-CD	41 ± 8	0.75 ± 0.03
HP-γ-CD	42 ± 6	0.57 ± 0.07

significant fluorescence suppression was observed, the relatively low aqueous solubility of this CD prevented data from being obtained at above 10 mM, thus the large range of data required to reach the plateau region of the curve was unobtainable, resulting in a larger relative error in the value of K in this case as compared to HP-β-CD. An excellent linear double reciprocal plot was obtained ($r = 0.999$). In the cases of γ-CD and HP-γ-CD, the observed fluorescence suppression was quite small, as can be seen in Figure 3; however, satisfactory fits to Equation (3) were still obtained. In these two cases, the double reciprocal plots were close to linear ($r = 0.989$ and 0.991 for γ-CD and HP-γ-CD, respectively), but did show some curvature at low CD concentrations. This might indicate the involvement of higher-order complexes for these two γ-CD's, but the relatively small degree of fluorescence suppression obtained at these concentrations (and thus the very large errors in the reciprocal values) makes this difficult to confirm.

Discussion

A significant decrease in 7MC fluorescence is observed in the presence of both β-CD and HP-β-CD, with similar F/F_o values of 0.69 and 0.66 in the presence of 10 mM of the two CD's, respectively, and the same F_∞/F_o fit values (i.e., the extrapolated maximum suppression when all 7MC guests are included in CD cavities) within experimental error (0.41 ± 0.06 and 0.37 ± 0.03). We propose that this decreased fluorescence is a result of inclusion of the 7MC molecule into the CD cavity, which provides a less polar environment than the aqueous solution and hence results in a significant reduction in the 7MC fluorescence. This polarity-dependence of 7MC fluorescence can be explained as follows [3, 14]. In a nonpolar medium, the energy of the $S_1(\pi\pi^*)$ state is similar to a nearby triplet ($n\pi^*$) state. This results in very efficient intersystem crossing (ISC), which competes with fluorescence, and results in a relatively low fluorescence quantum yield, ϕ_F. However, in a polar medium, the energy of the $S_1(\pi\pi^*)$ state is lowered below that of the triplet ($n\pi^*$) state, so that ISC can only occur to the $T_1(\pi\pi^*)$ state; this is much less efficient due to a significant energy gap, resulting in a larger ϕ_F. Thus, upon inclusion of 7MC into the CD cavity, the decreasing polarity of the 7MC environment results in a decrease in ϕ_F through an increase in the rate of ISC, and hence the observed decrease in fluorescence intensity, or fluorescence suppression. This effect allows for the study of the inclusion process via measurement of the decrease in 7MC fluorescence upon addition of CD. By contrast, the lack of effect of α-CD and HP-α-CD on 7MC fluorescence indicates that these cavities (*ca.* 5.7 Å) are too small to accommodate this guest, and hence a host–guest inclusion complex does not form.

There are two possible modes of inclusion of 7MC into β-CD to form a 1 : 1 host–guest complex, with insertion of either the carbonyl or the methoxy end first. It is difficult to establish a preference between these two modes, as there are oxygen atoms on both ends of 7MC, carbonyl on one end and methoxy on the other, which could undergo hydrogen bonding. Thus, there could be significant interaction between hydroxyl groups on the upper rim of the CD and the oxygen atoms on whichever end of the guest molecule is oriented towards the top of the cavity (i.e., either carbonyl or methoxy).

The observed similar effect of β-CD and HP-β-CD on 7MC fluorescence intensity is in marked contrast to the much greater effect of modified relative to parent CDs observed in various cases of fluorescence enhancement. [15–18] For example, in the case of the fluorescent probe 1,8-ANS, enhancements of 180 and 8.4 were observed in the case of 10 mM HP-β-CD and β-CD, respectively. [17] Similarly, in the case of the related probe 2,6-ANS, enhancements of 82 and 31 were observed in these two CDs, respectively. [18] This significantly increased enhancement by modified CDs as compared to the parent has been explained as a result of the replacement of some of the hydroxyl groups around the rim of the CD cavity by hydroxyalkyl groups; this is proposed to both extend the size of the CD cavity and to further reduce the cavity polarity [17, 18]. It is thus expected that HP-β-CD should provide a significantly greater fluorescence suppression of 7MC as compared to β-CD. This lack of a significant effect of the modification of the CD rims suggests that the 7MC molecule (or at least the aromatic fluorophore moiety) is more fully included in the β-CD cavity than in the case of the other fluorophores (i.e. very little of the 7MC is sticking out of the cavity),

so that substitution of hydroxypropyl groups around the CD rims would be expected to have little effect on the fluorescence of the included molecule. This is consistent with the more compact size of 7MC as compared to the two ANS guests, which have anilino and sulfonate groups attached to a central naphthalene moiety and hence can only be partially included.

Similarly, it has been observed that modified CDs in general bind guests more strongly than their unmodified parents [17–20]. Using the same examples as above, values of the binding constant K of 480 M^{-1} versus 80 M^{-1} were measured for 1,8-ANS in HP-β-CD and β-CD, and 7200 M^{-1} versus 1350 M-1 for 2,6-ANS in HP-β-CD and β-CD. [18] Again, the increased binding capacity of the modified CDs was explained to be a result of the extension of the cavity by the hydroxypropyl groups, which could hold the guest in place more effectively. This effect is also not observed for 7MC, as the values of K for β-CD (128 ± 32 M^{-1}) and HP-β-CD (120 ± 20 M^{-1}) are the same within experimental error. This observation supports the proposal that the 7MC molecule is well-contained within the β-CD cavity, thus addition of hydroxypropyl side arms has no significant effect on the stability of the inclusion complex.

Significant differences are however observed in the case of HP-γ-CD as compared to γ-CD, with maximum fluorescence suppressions (F_∞/F_o) of 0.57 ± 0.07 and 0.75 ± 0.03, respectively. In this case, the HP side chains are in fact having an effect, and result in a less polar cavity being experienced by the 7MC fluorophore. As mentioned above, there is some indication of the possible role of higher-order complexes in the case of γ-CD and HP-γ-CD; this may explain the effect of substitution in the case of γ-CD's which was absent in the case of β-CD's. Interestingly, however, there is no significant effect on the observed equilibrium constant (assuming 1 : 1 complexation), which is the same for both γ-CD and HP-γ-CD within experimental error (41 ± 6 and 42 ± 8 M^{-1}, respectively).

The significantly lower value of K obtained for 7MC in γ-CD and HP-γ-CD (*ca.* 40 M^{-1}) as compared with β-CD and HP-β-CD (*ca.* 120 M^{-1}) indicates that there is a better match between the size of 7MC and that of the β-CD cavity (*ca.* 7.8 Å) as compared with that of the γ-CD cavity (*ca.* 9.5 Å). Since the inclusion complex is held together by short-range intermolecular forces, this match in size is very important in determining the stability of the inclusion complex. While the HP side chains are effective at lowering the polarity experienced by 7MC in the case of the large γ-CD cavity, they do not contribute to binding of the 7MC guest inside this too large cavity.

The measurement of the decreased fluorescence for 7MC as the ratio F/F_o, as opposed to the difference $F - F_o$ [5, 6, 8, 9] (or simply F itself [10]) used in previous reports, has significant advantages. First, this value is independent of the fluorescent guest concentration, as well as instrumental parameters such as optical geometry and lamp intensity. Thus, while $F - F_o$ would be different as measured in different laboratories, or even on the same instrument on different days, the measured value of F/F_o is independent of these experimental parameters, and is thus a useful way of expressing the decrease in fluorescence. Furthermore, this value provides a relative measurement of the change in fluorescence quantum yield ϕ_F of the guest upon inclusion (assuming that inclusion does not affect the absorbance of the guest, and that the presence of the host does not significantly change the refractive index of the solvent). Thus the quantity F/F_o has direct photophysical significance which $F - F_o$ does not have.

We further assert that the term "fluorescence quenching" used in previous reports [5–7] to describe this observation of decreased fluorescence intensity upon inclusion into CDs is misleading, and suggest the better term "fluorescence suppression". Fluorescence quenching refers to an intermolecular interaction between the fluorophore and a quencher molecule, the latter of which de-activates the former by removing its excitation energy. The energy can be removed by direct collisional deactivation or by long-range energy transfer (Förster transfer), with the result being an additional non-radiative photophysical decay path for the fluorophore, and a concomitant decrease in fluorescence quantum yield. Alternatively, a chemical reaction can occur between the fluorophore and quencher, such as electron transfer or H atom abstraction, which also serves to depopulate the fluorophore excited state. In either case, this is mechanistically very different from what typically occurs in the case of a fluorophore which has become included in a CD cavity. In this case, the local environment of the included fluorophore is different than that of the free fluorophore in solution. If the fluorophore is polarity-sensitive, then the fluorescence parameters of the included fluorophore will be changed. In the case of 7MC, the fluorescence quantum yield decreases with decreasing polarity, as described above. This does not occur by deactivation of the 7MC excited state by a quencher molecule, but by the change in local environment. Thus, we feel that the term "fluorescence suppression", by analogy to the widely-used term "fluorescence enhancement" for the case of a guest which shows increased fluorescence upon inclusion, should be used instead of the term "fluorescence quenching". This is further supported by the fact that the effect of CD inclusion on cases of bimolecular fluorescence quenching involving a quencher other than the host have been investigated [21–25]. This is a very different process, in which inclusion of a guest into a CD helps reduce the efficiency of quenching by a third molecule. There has also been a report of the quenching of an included guest by a CD with a quencher (in this case viologen) tethered to it [26]. Furthermore, there has been a report of direct quenching of a guest by a cyclodextrin host: the fluorescence of a series of bicyclic azoalkanes is found to decrease upon inclusion into CDs as a result of H atom abstraction from the CD cavity [27]. Thus, the phenomenon of reduced fluorescence emission upon inclusion into a CD cavity resulting from the change in polarity of the environment should be referred to as fluorescence suppression, to distinguish this phenomenon from true cases of bimolecular quenching via energy transfer, electron transfer, or photochemistry.

Conclusions

7MC forms host–guest inclusion complexes with parent and modified β- and γ-CD in aqueous solution, which results in a reduction of the 7MC fluorescence intensity, a phenomenon we describe as fluorescence suppression. Quantitative measurement of the fluorescence suppression as the ratio F/F_o of the total fluorescence in the presence and absence of the CD provides an instrument-independent value, which is directly related to the effect of inclusion on the fluorescence quantum yield of the guest. Furthermore, this ratio F/F_o can be used to determine the association constant K for the inclusion process. In the case of HP-β-CD, a value of $K = 120 \pm 20\ \mathrm{M}^{-1}$ was obtained; in the case of the parent β-CD, a similar value for $K = 128 \pm 32\ \mathrm{M}^{-1}$ was obtained. A similar degree of fluorescence suppression was also observed for these two β-CD's. This lack of effect of CD modification indicates that 7MC must be deeply included in the CD cavity, with no part of the fluorophore protruding significantly beyond the CD rim. In the cases of γ-CD and HP-γ-CD, a significantly lower value of K of *ca.* 40 M^{-1} was observed. This reflects a poorer match in size between 7MC and γ-CD *versus* β-CD. While there was no effect of HP substitution on the equilibrium constant in the case of γ-CD, there was a significant effect on the fluorescence suppression observed, indicating that the HP groups are having a significant effect on the polarity experienced by 7MC in the case of the large γ-CD, unlike the case of the better size match with the β-CD cavity.

Acknowledgements

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