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Lumichrome complexation by cyclodextrins: Influence of pharmaceutical excipients

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Complexation of the model drug lumichrome by 2-hydroxypropyl- β -cyclodextrin (HP β CD), the most widely used cyclodextrin derivative in pharmaceutical preparations, was investigated in this study. The influence of frequently used pharmaceutical excipients, i.e. alcohols (ethanol, glycerol, propylene glycol), buffers (phosphate, citrate) and tonicity modulators (NaCl, MgCl₂) was evaluated by phase solubility, absorption and fluorescence emission spectra and fluorescence lifetime studies. Further, complex formation constants and fluorescence quantum yields were calculated. The formation of a 1:1 complex was indicated by phase solubility studies. The shape of the absorption and emission spectra for lumichrome was nearly independent of dissolution medium. The intensity of the absorption peak was slightly decreasing by the addition of HP β CD, which indicates formation of an inclusion complex of lumichrome in the ground state. The intensity of the fluorescence emission peak (i.e. fluorescence quantum yield) was also steadily decreasing by the increase in HP β CD concentration. Monoexponential fluorescence decay was obtained in the absence of cyclodextrin. In the presence of cyclodextrin, bi-exponential decays were observed in all aqueous vehicles with the exception of plain water or samples containing salts. The longest decay time corresponds to the lifetime of free (uncomplexed) lumichrome, while the shortest decay time was attributed to the excited state of the complexed alloxazine form of lumichrome. The selected excipients influence the complexation constant and the lumichrome excited state deactivation pathways to various extents.

1. Introduction

Cyclodextrins (CD) are cyclic oligosaccharides, composed of glucopyranose units, with a cavity in the centre. In a pharmaceutical context, they are mainly used to increase the aqueous solubility, bioavailability and stability of drug molecules. In addition, they can prevent drug-drug or drug-excipient interactions within a formulation as well as reduce drug irritation *in vivo* after topical and oral administration. 2-Hydroxypropyl- β -cyclodextrin (HP β CD) is the most widely used cyclodextrin derivative in pharmaceutical preparations due to high water solubility and low toxicity. It is pending for approval by FDA for intravenous administration (FDA 2010). The strength of complexation between the drug molecule and the cyclodextrin is reflected by the binding constant for inclusion of the hydrophobic “guest” molecule in the cavity of the water soluble cyclodextrin “host”. The complexation is influenced by a number of factors like shape and size matching between the guest and the CD cavity, and hydrophobic interactions. A pharmaceutical preparation will in most cases contain one or several excipients in addition to the drug-CD complex. Addition of a “third party” is likely to have impact on the drug-CD complexation and may alter the solubility, bioavailability and stability of the preparation compared to a simple drug-CD model system. Understanding the role of additives is crucial in the preformulation work.

Lumichrome has been used as a model drug in the present work. There is a current interest in alloxazine and isoalloxazine derivatives as potential photosensitizers in antibacterial photodynamic therapy (aPDT) (Bouillaguet 2008). These compounds generate singlet oxygen by energy transfer from the triplet state with a rather high efficiency (Sikorski et al. 2001). Further, lumichrome, which is a major biodegradation and photodecomposition product of riboflavin, is suitable as an indicator of changes in the microenvironment. This substance undergoes photo-induced tautomerization to form an isoalloxazine structure (Fig. 1). The process is accompanied by spectral changes that can be observed both in the UV-Vis range and fluorimetrically (Sikorska et al. 2003a, b; Song et al. 1974). The tautomerization is strongly dependent on the microenvironment (Miskolczy and Biczók 2005). It is well known that CD-complexed drugs can exhibit photochemical reactions which are quite different from what is observed in homogenous solution (Bortolus and Monti 1996), but the influence of excipients on the photochemistry of CD-complexed drugs is less investigated. In the present work, we therefore wanted to investigate the effect of frequently used pharmaceutical excipients on the lumichrome-CD complex formation. HP β CD was the cyclodextrin of choice, and the complexation was studied in aqueous media containing various amounts of alcohols (i.e. ethanol, glycerol, propylene glycol), various buffer systems (i.e. phosphate, citrate) or

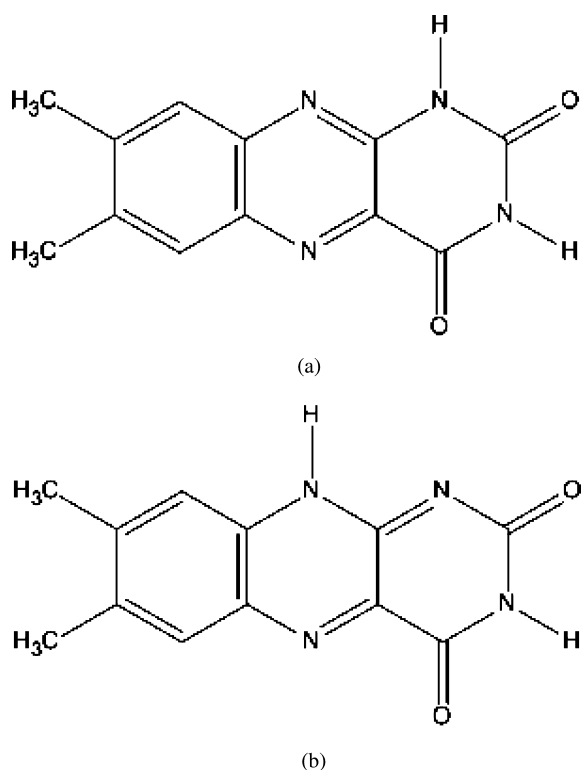


Fig. 1: Lumichrome (a) and its tautomer isalloxazine form (b)

different tonicity modulators (i.e. NaCl, MgCl₂) at various ionic strengths.

2. Investigations, results and discussion

2.1. Complex formation constants and complexation efficiency determined from phase solubility studies

Phase solubility studies showed a linear increase in dissolved lumichrome as a function of HPβCD concentration in the range 0-10% w/v. The linear increase in dissolved drug indicates the formation of a 1:1 complex. The stability constant ($K_{1:1}$) of the lumichrome/CD complex was calculated from Loftsson et al. (1999):

$$K_{1:1} = \text{slope}/[S_0(1 - \text{slope})] \quad (1)$$

where slope is the calculated slope of the linear phase-solubility diagram and S_0 is the intrinsic solubility of lumichrome determined in the aqueous complexation media in the absence of CD. Further, the solubilizing (complexation) efficiency (CE) of the cyclodextrin in a particular aqueous vehicle is also an important parameter to be determined in the preformulation phase in order to optimize solubilization. The complexation efficiency can be determined from Loftsson et al. (2005):

$$CE = S_0 K_{1:1} \quad (2)$$

The S_0 and $K_{1:1}$ values determined in the various aqueous vehicles are presented in Table 1. The calculated CE are presented in Fig. 2. The S_0 of lumichrome is highest in 1% (v/v) ethanol; i.e. the value slightly exceeds plain water. All the other vehicles seem to cause a small reduction in S_0 , and the effect is most pronounced in phosphate- and citrate buffer and in the presence of magnesium chloride. Further, the complexation constant $K_{1:1}$ is apparently not influenced by the addition of citrate buffer, sodium chloride and glycerol at the given concentrations. In the case of phosphate buffer, the complexation constant increases slightly, while a small decrease is observed in the presence of magnesium chloride, propylene glycol and ethanol. The latter is

Table 1: Intrinsic solubility (S_0) of lumichrome and stability constant ($K_{1:1}$) for lumichrome-CD complexes in aqueous vehicles ($n = 3$). The stability constant is determined from samples containing 0, 1, 3, 5, 7 and 10% w/v of HPβCD, respectively, and the average S_0 of the samples was used

Excipient	S_0 (M) (deviation in %)	$k_{1:1}$
Water	1.9×10^{-5} (7)	382
Phosphate	1.2×10^{-5} (11)	452
Citrate	1.2×10^{-5} (3)	385
NaCl	1.8×10^{-5} (1)	386
MgCl ₂	1.2×10^{-5} (6)	335
EtOH	2.4×10^{-5} (13)	256
Prop glycol	1.4×10^{-5} (9)	359
Glycerol	1.8×10^{-5} (8)	388

Phosphate = 0.05M phosphate buffer pH 5, $\mu = 0.05$; Citrate = 0.05 M citrate buffer pH 5, $\mu = 0.125$; NaCl = sodium chloride, $\mu = 0.05$; MgCl₂ = magnesium chloride, $\mu = 0.05$; EtOH = 1 % ethanol; Prop glycol = 1 % propylene glycol; Glycerol = 1 % glycerol

causing the most pronounced effect on the complexation constant of the excipients investigated. However, all the excipients seem to reduce the complexation efficiency of HPβCD compared to plain water (Fig. 2). The effect is most pronounced for magnesium chloride, which reduces the complexation efficiency to nearly half the value of plain water. It is reported that salts, particularly at high concentration, sometimes can lower S_0 through salting out effects and further decrease the complexation efficiency (Loftsson et al. 1999). This might (partly) explain the observed effect of MgCl₂. Concerning organic salts and acids, they may form non-covalent or ion-pair associations with a basic drug combined with the CD which might increase the complexation (Loftsson et al. 1999). Lumichrome in the ground state can undergo deprotonation in two steps leading to the formation of the mono- and dianion, respectively. The pK_a values for these deprotonation steps are determined to ≈ 8 and >12 (Sikorska et al. 2003b). Lumichrome would therefore appear in the neutral form at pH 5 and is unlikely to form such ion pair associations with the selected organic buffer salts. The observed decrease in CE value in the presence of phosphate and citrate is not so easily explained, but is mainly caused by a reduction in intrinsic solubility as seen from Table 1.

Alcohol co-solvents can have a stabilizing or destabilizing effect on the inclusion of a drug molecule into the CD cavity (Huang et al. 1992). In the first case, higher order complexes are formed and the van der Waal interaction between host and guest is enhanced. In the second case, the co-solvent can expel the probe

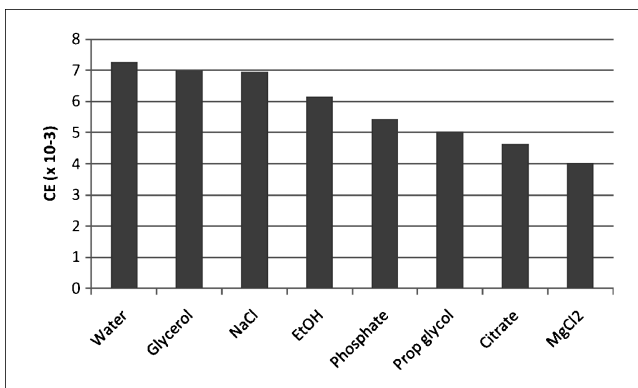


Fig. 2: Complexation efficiency (CE) × 10⁻³ calculated for lumichrome-CD complexes in aqueous vehicles ($n = 3$). Phosphate = 0.05 M phosphate buffer pH 5, $\mu = 0.05$; Citrate = 0.05 M citrate buffer pH 5, $\mu = 0.125$; NaCl = sodium chloride, $\mu = 0.05$; MgCl₂ = magnesium chloride, $\mu = 0.05$; EtOH = 1 % ethanol; Prop glycol = 1 % propylene glycol; Glycerol = 1 % glycerol

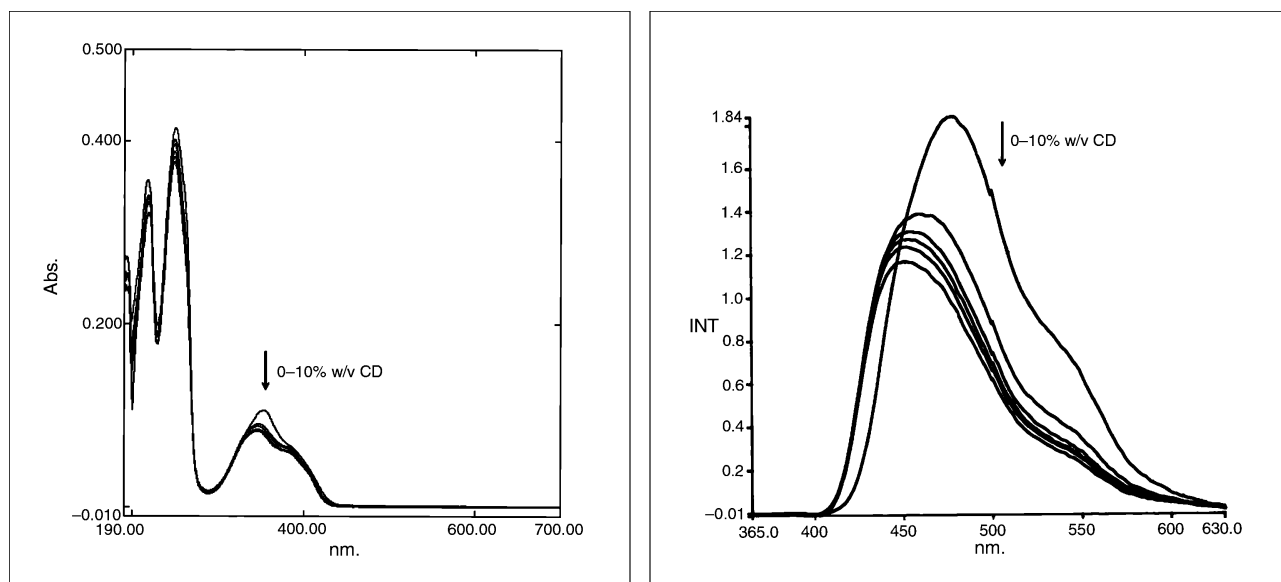


Fig. 3: Absorption (left) and fluorescence emission (right) spectra of lumichrome in samples containing NaCl ($\mu = 0.05$) as a function of cyclodextrin concentration. The spectral intensities are decreasing from 0 %w/v HP β CD (highest value) through 1, 3, 5, 7 and 10 % w/v HP β CD (lowest value), as indicated by arrows

from the cavity in a competitive binding scheme or decrease the bulk solvent polarity to destabilize the inclusion complex. The overall effect of the co-solvent depends on the alcohol concentration and chain length / bulkiness. For the individual formulations (e.g. combination of drug substance, CD and type of alcohol) an optimum co-solvent concentration is likely to exist (Nelson et al. 1989). In the present study, the complexation efficiency of lumichrome within the HP β CD cavity was decreased by addition of 1% alcoholic co-solvent in the order propylene glycol > ethanol > glycerol (Fig. 2). Propylene glycol lowers the intrinsic solubility of lumichrome and has also a weak reducing effect on the complexation constant (Table 1); both effects will contribute to the reduction in complexation efficiency (Eq. 2). Ethanol on the other hand, increases the intrinsic solubility of lumichrome but might be too small to occupy the vacant zone of the CD cavity to form a ternary complex (Hamai 1989). It might even compete with or expel lumichrome from the cavity. The latter effects are indicated by a decrease in the complexation constant, which suggests a weaker interaction with the CD in the presence of ethanol (Mrozek et al. 2002). Glycerol has little effect on both the intrinsic solubility of lumichrome and on the complexation constant (Table 1) and thereby a small influence on the complexation efficiency. In this context, glycerol seems to be the most inert of the excipients investigated.

2.2. Absorption and fluorescence emission spectra and fluorescence quantum yields

The absorption and fluorescence emission spectra were obtained, and the fluorescence quantum yields were calculated for lumichrome in various aqueous vehicles containing 0, 1, 3, 5, 7 and 10% w/v of HP β CD, respectively. The shape of the absorption and emission spectra looked almost identical irrespectively of excipient present in the aqueous vehicle. Figure 3 shows a representative example of the spectra as a function of CD concentration. Table 2 shows the results for samples containing 0, 1 and 10 %w/v CD, respectively. The wavelength of the absorption and emission maxima is nearly unaffected by the type of aqueous vehicle in the absence of CD. Bathochromic shifts are however, observed by addition of cyclodextrin. The shift in emission wavelength is larger than the shift in absorption wavelength, and both shifts are dependent on the CD concentration as illustrated by the data for the lowest and highest CD concentration

(Table 2). Further, the intensity of the absorption peak is slightly decreasing by the addition of CD (Fig. 3). This indicates the formation of an inclusion complex of lumichrome in the ground state. The intensity of the fluorescence emission peak is steadily decreasing by the increase in CD concentration as demonstrated by a decrease in the fluorescence quantum yield (Table 2). There have been relative few reports of decreased fluorescence of guest molecules upon cyclodextrin inclusion in aqueous solution, but one example referred to in the literature is just lumichrome which thereby confirm our observations (Sakar et al. 1995; Wagner et al. 2003). It is further reported that HP β CD might be a stronger quencher of fluorescence than the corresponding unsubstituted cyclodextrins (Fraiji et al. 1994). Lumichrome possesses close-neighborly n, π^* and π, π^* singlet excited states (Sikorska et al. 2004a). In the less polar CD cavity it may be expected that n, π^* is the favoured first excited singlet state. This low-lying n, π^* state may provide efficient non-radiative decay channels thereby lowering the fluorescence quantum yield of lumichrome in the presence of CD (Sikorska et al. 2004b). It is however, apparent that the lumichrome inclusion complex emits some fluorescence which is further emphasized by the lifetime studies (Sec. 2.5).

2.3. Effect of ionic strength

The absorption and fluorescence emission spectra were obtained, and the fluorescence quantum yields were calculated in samples containing NaCl and MgCl₂ as a function of ionic strength. To the samples was added CD at concentrations 0, 1, 3, 5, 7 or 10 %w/v. The results obtained at 0, 1 and 10 %w/v CD are presented in Table 3. Consistent with the observations made for the various vehicles in Sec. 3.2, the shape of the absorption and emission spectra and the wavelength of the absorption and emission maxima were nearly unaffected by the ionic strength in the absence of CD while bathochromic shifts were observed by addition of cyclodextrin. The shifts were dependent on the CD concentration as illustrated by the data for the lowest and highest CD concentration (Table 3). Further, the intensity of the absorption peak is slightly decreasing by the addition of CD. The intensity of the fluorescence emission peak (i.e. quantum yield) is also steadily decreasing by an increase in ionic strength. The effect is most pronounced in the absence of CD (Table 3). This can probably be ascribed to the quenching of the free (unbound) fraction of lumichrome. The per cent decrease in fluorescence

Table 2: Absorption (λ_{abs}) and emission (λ_{em}) maxima and fluorescence quantum yield (φ_{fl}) of lumichrome samples in various aqueous vehicles containing 0, 1 or 10 % w/v of 2-hydroxypropyl- β -cyclodextrin (n = 3)

Excipient	Water			1% w/v CD			10% w/v CD		
	λ_{abs}	λ_{em}	φ_{fl}	λ_{abs}	λ_{em}	φ_{fl}	λ_{abs}	λ_{em}	φ_{fl}
Water	351	477	0.029 \pm 0.001	349	461	0.022 \pm 0.001	344	453	0.021 \pm 0.001
Phosphate	352	478	0.030 \pm 0.001	349	461	0.021 \pm 0.001	350	451	0.019 \pm 0.001
Citrate	351	480	0.028 \pm 0.001	347	464	0.020 \pm 0.001	344	451	0.018 \pm 0.001
NaCl	354	479	0.020 \pm 0.001	349	463	0.019 \pm 0.001	344	452	0.017 \pm 0.001
MgCl₂	354	478	0.023 \pm 0.001	348	462	0.020 \pm 0.001	347	452	0.018 \pm 0.001
EtOH	352	477	0.026 \pm 0.002	347	467	0.018 \pm 0.001	345	451	0.016 \pm 0.001
Prop glycol	353	476	0.029 \pm 0.001	348	465	0.022 \pm 0.001	346	449	0.018 \pm 0.001
Glycerol	353	477	0.029 \pm 0.001	348	465	0.021 \pm 0.001	345	453	0.016 \pm 0.001

Phosphate=0.05M phosphate buffer pH 5, μ =0.05; Citrate=0.05 M citrate buffer pH 5, μ =0.125; NaCl=sodium chloride, μ =0.05; MgCl₂=magnesium chloride, μ =0.05; EtOH=1 % ethanol; Prop glycol=1 % propylene glycol; Glycerol=1 % glycerol

quantum yield over the range μ = 0.05-0.5 at a certain CD concentration is virtually independent on the type of quenching ion; i.e. the reduction in the absence of CD was 75% and 70% for NaCl and MgCl₂ respectively, compared to 32% and 35% respectively, at 1 % w/v CD; and 12 % and 28 % respectively, at 10 % w/v CD (Table 3). In conclusion, the ionic strength apparently influences the ratio between the deactivation pathways of excited lumichrome. In this context, the presence of CD makes the photosensitizer less sensitive to variations in ionic strength.

2.4. Effect of alcohol concentration

The concentration of ethanol, propylene glycol and glycerol was varied between 1% and 5% in samples containing 0, 1, 3, 5, 7 and 10 % w/v CD. The same effects as reported in Table 2 for vehicles containing 1 % of the alcohols were confirmed in the case of 3 % and 5% of the alcohols (data not shown). The absorbance of the samples did however, increase steadily as a function of increasing alcohol concentration independent of the presence of CD (data not shown). This reflects probably a general increase in lumichrome solubility in the vehicle by a decrease in vehicle polarity. Because the presence of alcoholic cosolvent decreases the complexation efficiency as described above (Sec. 2.1), an increased alcohol concentration is likely to increase the amount of free (uncomplexed) lumichrome in the preparation.

2.5. Fluorescence lifetime

The fluorescence lifetime of lumichrome in the various aqueous excipients in the absence and presence of cyclodextrin is pre-

sented in Table 4. The fluorescence lifetime in water is consistent with previously published results (Sikorska et al. 2004a; Miskolczy et al. 2009). This lifetime keeps constant upon addition of buffer or alcoholic cosolvents in the absence of cyclodextrin. The observed lifetime is however, shortened upon addition of salts (i.e sodium or magnesium chloride). A bi-exponential decay is observed in the presence of cyclodextrin in all aqueous vehicles with the exception of plain water or samples containing salts (Table 4). The longest decay times correspond to the lifetimes of lumichrome in plain water, i.e. the lifetime of free (uncomplexed) lumichrome. The decay was detected at 425 nm where the alloxazine form of lumichrome emits and the contribution from the isoalloxazine form is neglectable (Sikorska et al. 2005). The shortest decay time ($\tau \sim 0.8$ -1.2 ns) was therefore attributed to the excited state of the complexed alloxazine form of lumichrome. This is consistent with previous reports on complexed lumichrome (Miskolczy et al. 2009), although the lifetime range was at the border of the detection limit of our system. The isoalloxazine form of lumichrome is reported in a previous work to coexist with the alloxazine form in the ground state supramolecular complex of lumichrome with cucurbit[7]uril (Miskolczy et al. 2009). In that case, the complexed isoalloxazine had a fluorescence lifetime of 5.1 ns detected at ≥ 440 nm. Further, the lifetime of uncomplexed isoalloxazine has been determined at 550 nm to ~ 7.4 ns under acidic conditions and ~ 6.3 ns in ethanol (Sikorska et al. 2005); two experiments that we did reproduce with similar results. Lumichrome is unable to tautomerize neither in the excited nor in the ground states in plain water and no evidence has been found for the tautomerization in the presence of β -CD (Miskolczy et al. 2009). In the present study, the decay was also

Table 3: Absorption (λ_{abs}) and emission (λ_{em}) maxima and fluorescence quantum yield (φ_{fl}) of lumichrome samples containing 0, 1 or 10 % w/v of 2-hydroxypropyl- β -cyclodextrin as a function of ionic strength (n = 3)

Excipient	Water			1% w/v CD			10% w/v CD		
	λ_{abs}	λ_{em}	φ_{fl}	λ_{abs}	λ_{em}	φ_{fl}	λ_{abs}	λ_{em}	φ_{fl}
NaCl μ=0.05	354	479	0.020 \pm 0.001	349	463	0.019 \pm 0.001	344	452	0.017 \pm 0.001
NaCl μ=0.10	352	477	0.015 \pm 0.001	347	456	0.016 \pm 0.001	347	450	0.016 \pm 0.001
NaCl μ=0.30	353	480	0.008 \pm 0.001	347	456	0.014 \pm 0.001	346	451	0.015 \pm 0.001
NaCl μ=0.50	354	480	0.005 \pm 0.001	349	450	0.013 \pm 0.001	345	448	0.015 \pm 0.001
MgCl₂ μ=0.05	354	478	0.023 \pm 0.001	348	462	0.020 \pm 0.001	347	452	0.018 \pm 0.001
MgCl₂ μ=0.10	352	478	0.018 \pm 0.001	348	461	0.018 \pm 0.001	347	450	0.017 \pm 0.001
MgCl₂ μ=0.15	353	478	0.017 \pm 0.001	346	457	0.017 \pm 0.001	347	450	0.016 \pm 0.001
MgCl₂ μ=0.30	352	476	0.011 \pm 0.001	348	459	0.014 \pm 0.001	347	452	0.016 \pm 0.001
MgCl₂ μ=0.50	353	477	0.007 \pm 0.001	347	455	0.013 \pm 0.001	348	450	0.013 \pm 0.001

NaCl = sodium chloride; MgCl₂ = magnesium chloride

Table 4: Fluorescence lifetime (τ) of lumichrome in nanoseconds (ns) in the absence of cyclodextrin and in the presence of 3% w/v cyclodextrin (n=3). The lifetime was detected at emission 425nm

Excipient	Fluorescence lifetime (τ)(ns) in the absence of cyclodextrin	Fluorescence lifetime (τ)(ns) in 3%w/v of cyclodextrin
Water	2.8 ± 0.1	1.2 ± 0.1
Phosphate	2.4 ± 0.1	0.8 ± 0.1 2.1 ± 0.3
Citrate	2.5 ± 0.1	1.0 ± 0.1 2.6 ± 0.6
NaCl	0.9 ± 0.1	0.8 ± 0.1
MgCl₂	1.1 ± 0.1	0.9 ± 0.1
EtOH	2.7 ± 0.1	0.7 ± 0.1 2.6 ± 0.1
Prop glycol	2.6 ± 0.1	0.8 ± 0.1 2.5 ± 0.2
Glycerol	2.6 ± 0.1	0.8 ± 0.1 2.5 ± 0.2

Phosphate = 0.05M phosphate buffer pH 5, μ = 0.05; Citrate = 0.05 M citrate buffer pH 5, μ = 0.125; NaCl = sodium chloride, μ = 0.3; MgCl₂ = magnesium chloride, μ = 0.3; EtOH = 1 % ethanol; Prop glycol = 1 % propylene glycol; Glycerol = 1 % glycerol

recorded at 550 nm, i.e. where the isoalloxazine form is expected to emit strongly compared to the alloxazine form (Miskolczy and Biczók 2005). No emitting component with lifetime in the range ~ 5-7 ns could be observed except for samples containing 1 % glycerol where a longer emitting component was observed (τ ~ 6.1 ns) in the presence of HP β CD. The difference between the decay time of free lumichrome and the longer emitting component was however, insufficient for a good resolution of the coexisting emissions. It is therefore difficult to predict whether the longer emission originates from traces of the complexed isoalloxazine form of lumichrome. In that case, the supramolecular complex formation with HP β CD in the presence of glycerol would have promoted tautomerization of lumichrome in a similar way to cucurbit[7]uril complexation. The presence of glycerol did however, seem to have minimal effect the lumichrome-CD complex formation (Table 1, Fig. 2). Further investigations should therefore be carried out to confirm the presence of the isoalloxazine form of lumichrome in this system. The decay time of lumichrome in the presence of salts but in the absence of CD is very similar to the decay time observed for the complexed lumichrome (i.e. τ \approx 0.8-1.1 ns in both cases) (Table 4). The lifetime in the presence of both salt and CD can therefore be described by a mono-exponential decay function although the signal most likely consists of a mixture of complexed and free lumichrome. A Stern-Volmer plot of F_0/F vs. [HP β CD] where F_0 and F is the fluorescence intensity in the absence and presence of CD respectively, showed deviation from linearity ($R^2 < 0.99$) at $\mu > 0.1$ (data not shown). This further emphasizes the presence of two emitting species with similar lifetime in the presence of magnesium- or sodium chloride. Fluorescence quenching is therefore not a suitable method for determination of complexing constants in those systems in spite of the apparent mono-exponential fluorescence decay.

3. Experimental

3.1. Determination of complex formation constants from phase solubility studies

Complexes of lumichrome (Aldrich; used as received) with cyclodextrin were prepared by addition of an excess of lumichrome to an aqueous cyclodextrin solution. The suspension formed was equilibrated for one week under continuous agitation (150 rpm) at ambient temperature. The samples

were filtered (Spartan 13/0.45 RC) prior to analysis by HPLC (Shimadzu chromatographic system; pump: LC-9A, autosampler: SIL-9A, detector: SPD-10A operated at 374 nm, recorder: C-R5A Chromatopac). The stationary phase was Nova-Pak[®] C₁₈, 4 μ m, 3.9 \times 150 mm column (Waters). The mobile phase consisted of water/methanol (13:8). The retention time of lumichrome was ~ 11 min. The lower detection limit ($3 \times$ the noise at the baseline) was 5.1×10^{-7} M. A standard curve was prepared in the concentration range 8.3×10^{-7} M – 4.1×10^{-6} M (reg > 0.999; RSD \leq 2 % for 4.1×10^{-6} M).

The cyclodextrin used was 2-hydroxypropyl- β -CD (HP β CD, Cavalot[®] W7 HP Pharma, Wacker). The water content of the CD was determined prior to use (Moisture Analyzer MA 30, Satorius) and corrected for in the further calculations. The CD concentrations (% w/v) used in the phase solubility study were 0, 1, 3, 5, 7 and 10.

3.2. Aqueous vehicles

The aqueous vehicles consisted of distilled and filtered water without any additives or in the presence of phosphate buffer (0.01 M, μ = 0.01; 0.05 M, μ = 0.05), citrate buffer (0.01 M, μ = 0.025; 0.05 M, μ = 0.125), sodium chloride (μ = 0.05, 0.1, 0.3, 0.5), magnesium chloride (μ = 0.05, 0.1, 0.15, 0.3, 0.5), ethanol (1, 3 or 5% v/v), glycerol (1, 3 or 5% v/v) or propylene glycol (1, 3 or 5% v/v), respectively.

3.3. Absorption measurements

The absorption spectra (190-700 nm) were recorded on a UV-2101PC UV-Vis scanning spectrophotometer or a UV-2401PC, UV-Vis recording spectrophotometer; both from Shimadzu.

3.4. Fluorescence emission spectra and quantum yield

The fluorescence emission spectra and quantum yields ($\lambda_{ex} = \lambda_{abs}$ as given in Table 2) were measured by use of a Luminescence spectrometer, LS50B (Perkin Elmer). The sample temperature was kept constant at 25°C \pm 0.1°C (Grant water bath W 14). The fluorescence quantum yields were determined from the integrated fluorescence spectra by using the quantum yield of quinine sulfate (Fluka) in 0.05 M H₂SO₄ excited at 344 nm as a reference value ($\varphi_{ref} = 0.51$) (Velapoldi and Tønnesen 2004).

3.5. Fluorescence lifetime measurements

Life-time fluorescence measurements ($\lambda_{ex} = 337$ nm) were carried out on a PTI Modular Fluorescence System (C-71 Strobe Master System) using TimeMaster Pro[™] for Windows software (1 to 4 Exp Lifetime curve fitting program; n = 10). The excitation source was a pulsed lamp filled with hydrogen/nitrogen (1:1), pulse width ~ 1.8 ns obtained by use of a diluted sample of colloidal silica (Ludox CL, Aldrich) in distilled water.

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