Luminescence in organized media and supramolecular interactions: physicochemical aspects and applications

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Natural cyclodextrins are an example of simple organic molecules, which are able to complex many different molecules and especially water-insoluble organic ones. This property caught early the interest of researchers involved in the field of drug delivery and soon new modified cyclodextrins appeared with improved solubility or complexing efficiency. Analytical applications aiming at the determination of fluorescent drugs followed and revealed that not only was their luminescence maintained, but that it was often enhanced. The present review begins with a short introduction devoted to the analysis of the forces involved at the molecular level in the complexing process and to the nature of the various complexes observed. It then focuses on the most typical results obtained in the field of luminescence enhancement following complexation of molecules of biological interest. It is shown that analytical applications always benefit from an improved sensitivity as a result of this enhancement. Extensions of the scope of the technique such as the induced emission of non emitting chromophores (retinoids) as well as the enhancement in micellar solutions are also reviewed.

Introduction

Molecular luminescence of analytes in organized media or in supramolecular complexes of cyclodextrins

The photophysical and photochemical behaviour of molecules complexed to cyclodextrins (CD) is generally different from their behaviour in solution. The first observations in the field were qualitative and were related to the increased stabilisation of labile molecules, mostly drugs, against photodegradation when complexed to natural cyclodextrins [1]. Complexation by CD's was then the preferred subject for those involved in supramolecular chemistry and drug vectorisation techniques [2] as well as in separation techniques [3-5]. Stabilization of a molecule against photochemical degradation is still very important in the field of pharmaceutical sciences. A recent example is the stabilisation of promazine as an inclusion complex with β-cyclodextrin [6]. Other photochemists were attracted quite early to this field of research and initiated studies, which were mostly photophysical in nature [7,8]. The key observation was that the formation of supramolecular complexes of analytes with cyclodextrins (CDs) resulted in an increase of their fluorescence quantum yield or even in the appearance of room temperature phosphorescence (RTP) [9-12]. This enhancement has been used to increase the sensitivity and the selectivity in both luminescence and chromatographic techniques [13-15].

We will not say more about RTP with CDs as the subject will be developed in another contribution in the same issue of this Journal. However RTP is also observed in the solubilization of analytes in micellar colloïdal solutions and this field will be reviewed hereunder.

These properties have been used to develop analytical techniques, which combine the selectivity of complexation with an enhanced emission [16-19]. In this way an improvement in the detection limits is obtained in many separation techniques such as HPLC, capillary electrophoresis (CE) or planar chromatography. Furthermore these molecular interactions allow to reveal or to stabilize new molecular forms or to turn species which are normally non-emitting into luminescent ones. All these aspects are reviewed hereunder after a short summary on the nature of the intermolecular interactions, which lie at the heart of these effects.

Nature of the interactions between an analyte and a CD or a micelle

Without going into a thorough analysis of the origin of the interactions which exist between molecules in dense media which concern us here, it seems useful to recall the most important factors which should be taken into account when any type of luminescence, fluorescence or phosphorescence is involved.

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In a homogeneous medium, some of the spectroscopic properties of the analyte are related to the solvent polarity or polarisability, to its hydrophilic or hydrophobic character, to its dissociating power or to its ability to participate in hydrogen bonding [20]. These properties may be characterized by macroscopic parameters (refraction index, dielectric constant, pH, etc.). On a molecular or even an atomic scale, the description of the specific association of two or more molecules, as for instance in a cyclodextrin complex or a micelle, is based on the existence of several types of attractive or repulsive forces [21,22]. They mostly consist in Van der Waals and hydrophobic forces, electrostatic or dipolar interactions and hydrogen bonding. The term hydrophobic relates to situations in which Van der Waals forces play a dominant role when the guest tries to limit its contact area with water. Modelisation shows also that binding may also be associated to a release of the strain energy of the CD ring and must take into account the various solvent-accessible surface areas changes occuring during the association process [23].

A description based on an isolated supramolecular complex fully separated from its environment is not always a satisfactory one to explain its formation, especially when bulk water is in fact involved. As a matter of fact, the reason for this lies in the energy changes associated to the transfer of a molecule which has generally a low solubility in an aqueous phase towards the hydrophobic cavity of a CD or towards a micelle. These changes are weak compared to the total interaction energy of the molecular components of the system under study, determined before and after formation of the complex or tranfer to the micelle [23]. No attempt will be made for a systematic explanation of the observed phenomena in the rest of this review. This choice is of no consequence on the value of the observations based on fluorescence in the context of an analytical application.

Note that nothing will be said here about pure solvents, even if in most examples the fluorescent species complexed to a CD may be in contact with the solvent on a fraction of their surface.

Cyclodextrins and their inclusion complexes

The structure of cyclodextrins and the nature of supramolecular inclusion complexes

Natural cyclodextrins are cyclic oligosaccharides synthetized by bacteria. They contain 6, 7 or 8 α–D–glucopyranoses units interconnected by α-1,4 glycosidic bonds for α, β and γ cyclodextrins respectively. We will limit ourselves here to describe the elements of interest in the field of fluorescence. Readers interested in collecting more information about cyclodextrins, may refer to various books dedicated to these molecules [1,24,25].

In shape they look like a hollow truncated cone or a torus. Two secondary alcohols per glucopyranose unit are positioned on the rim of the larger opening whereas primary alcohols are located on the smaller one. The dimensions of the cavities of theses various natural cyclodextrins, from 0.60 to 0.64 nm, allow for the inclusion of a wide range of organic or inorganic guests (Fig. 1). β-CDs accomodate benzene and naphthalene derivatives, when larger γ-CD may include macrolides antibiotics [26] or steroids [27]. Of special interest to us, many molecules which have a low solubility in water form such inclusion complexes as a consequence of the hydrophobic character of the cavity. The solubilization of fluasterone [28] an analog of DHEA, or of ellipticine [29,30,31] are typical examples. The ellipticine

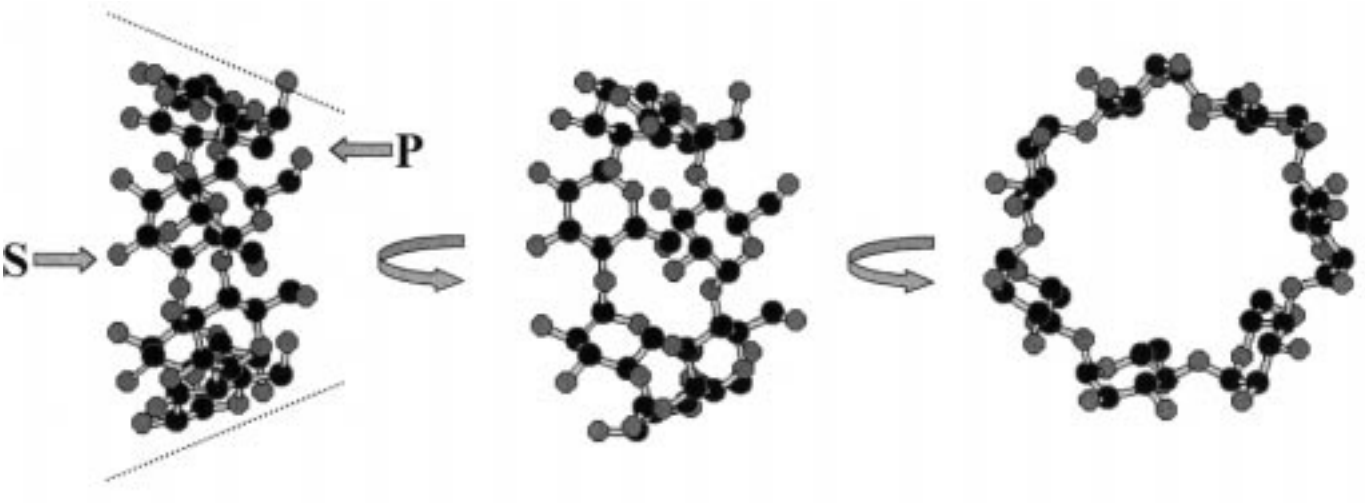

Figure 1. Ball and sticks representation of ß-cyclodextrin. For the sake of clarity, the hydrogen atoms are removed. The left view is a side view which brings out the conical shape of the cyclodextrin. The arrow S points to the secondary hydroxyls sitting on the large rim while the arrow P refers to the primary hydroxyls groups on the smaller rim. The center and right views result from a two-step rotation around a vertical axis. Note the position of the bridging oxygens which point towards the inside of the cavity.

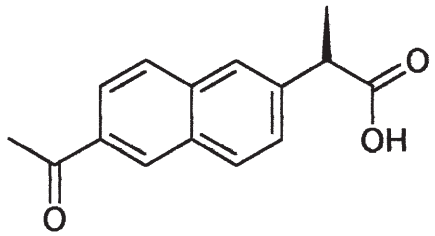

Figure 2. Naproxen.

and naproxen (Fig. 2) case illustrates also the fact that the ionic and neutral forms of the drug behave very differently in their affinity towards CD and the properties of their complexes. The benefit of inclusion is often twofold, as the complexes so obtained are soluble in an aqueous medium and the included species are protected against some of the unwanted effects of temperature, light, pH or interaction with molecular oxygen.

For a useful, if somewhat simplified description, the supposition can be made that the stabilisation of the complexes formed results from the lowering of the energy of the system due to the transfer of the molecule (or of the hydrophobic part of the molecule if the latter is a large one) from the aqueous phase towards the cavity. Actually, other factors come into play, the most important of them being the simultaneous transfer of most water molecules present in the cavity towards bulk water. These water molecules are initially bound *via* hydrogen bonds to the oxygen atoms in the cavity and to the hydroxylic groups on the rim [32] and the presence of some of them may be necessary to hold the complex together [33].

α–CD has a narrower cavity and is less flexible than β-CD. The cavity of γ–CD is larger than that of β-CD, but the energy stabilisation resulting from both the expulsion of water and the inclusion of the analyte (often referred to as the driving force) is larger for β-CD than for γ-CD. As a result, inclusion complexes formed with β-CD will generally be more stable [1]. It must be also pointed out that β-CD has a cavity size which accommodates most of the current analytes. Nevertheless, the dimensions of some molecules exceed the size of the cavity and they are only included in part. If they possess polar or hydroxyles groups, the latter will protrude towards the aqueous phase and may hydrogen bond to the hydroxyl groups of the rim. The hydrophobic character of the cavity of a CD is shown for example by the enhancement of the fluorescence of 1-8 ANS and 2-6 TNS in the presence of α or β–CD [34,35]. This will be developed further.

Modified cyclodextrins

Natural CDs, and specially β-CD, have an average to low solubility in water. To overcome this solubility barrier, many derivatives of natural CDs have been prepared [24,27]. They mostly derive from β-CD and the most popular are hydroxypropyl-β-cyclodextrin (HP-β-CD), (2,6-di-O-méthyl)-β-cyclodextrin (DM-β-CD) and (2,3,6-tri-O-methyl)-β-CD (TM-β-CD). Ionisable CDs were prepared such as for instance sulphobutylether-β-cyclodextrin. Exotic cyclodextrins were also synthetized for specific purposes: β-CD polymers [36], multichromophoric cyclodextrins [37] or bis-naphtylsulphonyl-βCD [38] and bis-pyrenyl-βCD [39] to detect organic compounds by a guest responsive monomer or excimer fluorescence. A similar solution was devised by Narita *et al.* to sense endocrine-disrupting chemicals and their analogues by fluorescence pattern recognition. They use dansyl or anthranilate modified β- and γ-cyclodextrins which give an excimer fluorescence in the absence of analyte. When an analyte forms an inclusion complex, the amount of excimer to monomer emission changes and a factor derived from this change together with the intensity of the signals form a set of data characteristic of a given analyte [40]. Another type of modification, in which one to three sugars of the CD were chemically modified or replaced by different sugars, was carried out [41]. A control of the deformation of the cavity induced by these modifications is possible. It leads to a change in the association constants for an included analyte. Such an approach coupled to modelization could lead to a powerful method to design cyclodextrin for the separation of specific classes of molecules.

Different complexes may be obtained for a given analyte

Numerous preparation techniques are known to produce inclusion complexes but they need not be described here (see for instance [42]). The attention must be drawn to the fact that several complexes of different stœchiometries can be obtained depending on the conditions: for example (1:1), (1:2) (2:1) or (2:2) complexes have been observed. Complexes of stoechiometry (2:2) results often from dimerization of (1:1) complexes [43,44] Several of them may coexist in given conditions. Some are soluble and some are insoluble. Ternary complexes are also known. These are (1:2) or (2:1) complexes with 1 or 2 molecules of analyte respectively or (1:1:1) complexes containing one water or solvent molecule and the analyte. For instance pyrene and β-CD in the presence of alcohols may form ternary complexes of various stoechiometries [45]. Another class of ternary complexes in which pyrene and various CDs interacted with nonionic surfactants was described by Nelson *et al.* The study shows that lifetime is very sensitive to inclusion and can be used as well as the steady state emission enhancement to follow complexation [46]. An interesting case of ternary complex concerns the addition of a third halogenated molecule (the halogen being Br or I) to a (1:1) complex of an analyte and CD, when the analyte does not emit or emits weakly. The effect of the added compound is to induce an efficient heavy-atom effect whereby the guest becomes phosphorescent. This case of RTP was nicely introduced for PAH and heterocyclic compounds by Scypinsky *et al.* using dibromoethane [9,47,48].

Insoluble infinite string-like sequences with a $(1:1)_n$ stœchiometry have been observed for diphenylhexatriene (DPH) [49] and for all-trans retinal [50]. They have been characterized by DSC, NMR, X-ray diffraction and AFM. A modelisation study of the interaction gives some hints as to the structure of such strings [51]. Even for a defined stœchiometry, let us say (1:1), the possibility exists for an included analyte with some conformational freedom to display different geometry [52-54]. The dynamics of such an invited analyte has been studied in the case of aryl alkylketones [55]. The effect of various alkyl chain lengths substituting the same naphthalimide nucleus was analyzed for the inclusion complexes with β-cyclodextrin [56]. The author concludes that a conformational control is exerted by the CD cavity since the emission intensities and the spectra fine structure are dramatically influenced by the chain length. The better-resolved spectra correspond to the longest C12 and C18 chains which, are folded and included together with the chromophore in the cavity.

A very effective restriction to rotational mobility induced by inclusion in a CD has also been observed for the photochemistry of molecules like cinnamic acid derivatives, stilbenes or ketones [57]. Similar effects on the photoisomerization and fluorescence properties of aromatic norbomadiene derivatives were reported following the formation of inclusion complexes with β-cyclodextrin [58]. The fact that a cavity is large enough to accomodate two chromophoric molecules has resulted in much activity related to the obtention of included excimers or controlled dimerization. This is the case for the formation of pyrene excimers from 10^{-6} M solutions of pyrene in water when γ-CD (final concentration 10^{-6} M) is added [59]. The addition of ß-CD or α-CD has no effect. Further addition of γ-CD to the (2:1) complexe favours another (1:2) complexe of lower affinity. As an example of controled photochemistry, a study of the dimerization of anthracene sulphonate by Tamaki *et al.* is very instructive. It shows that not only is dimerization accelerated 10-fold in water in presence of CDs, but also that the selectivity of the dimerization is affected by the nature of the CD. In the (1:2) γ-CD complex, the configuration of the dimer is the same than for the excimer in solution without CDs [60]. With β-CD, a (1:2) complex results which leads to a dimer with a different configuration.

The last point to emphasize is that complexes may be obtained in mixtures of water and organic solvents such as DMF, acetonitrile, DMSO [61]. In these solutions, the percentage (v/v) of solvent may reach 60 % in the case of DMF and DMSO. The solubility of the CDs is often the limiting factor. Usually, with small molecules, one molecule of solvent may be included to form a ternary complex [62].

What it takes to fully characterize one inclusion complex

The characterisation of a complex typically requires an array of techniques from physical chemistry. The traditional methods include one or several of the following: NMR, IR, Raman, DSC and TGA, AFM, XRD, neutron diffraction [63], electronic absorption and fluorescence.

The stœchiometry and the stability constants for the complexes are often obtained using a spectrophotometric or spectrofluorimetric method based on the Bénési-Hildebrand description [64]. If this procedure has often been used as described in the original publication [59,65-67], better results are obtained with variants of this method [68,69]. HPLC provides another way for obtaining the complexation constants of analytes with CDs [70,71]. With such chromatographic methods, the association constants of the guest: CD complexes may be obtained even if the spectroscopic changes resulting from the inclusion are absent or not significative. Furthermore, these techniques allow clearly following the effect of additives such as tert-butyl alcohol on the stoechiometry and apparent formation constants of the complexes. Some artefacts linked to deviation from the Beer-Lambert law have been shown to affect a chromatographic method using CD in the determination of tolfenamic acid using electronic absorption for the detection step [72]. In the case of fluorescent analytes, such artefacts could also affect the emission and the HPLC method used by the authors could be adapted to alleviate the accuracy problems due to this type of anomalous effects.

Applications of CDs

Solubilization of hydrophobic molecules

Solubilization of hydrophobic drugs by CDs is a widely used technique. It has been applied for instance to such molecules as vitamines A, D1 and K3, metronidazole, nitrazepam, phenothiazines derivatives [6], prostaglandins E (Fig. 3), retinoic acid.

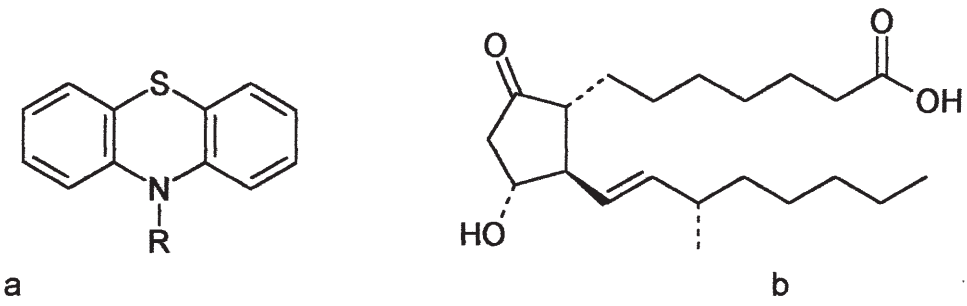

Figure 3. (a) Phenothiazines: in drugs, R is often an alkylamine substituent; (b) Prostaglandin E1.

In the latter instance it increases the biodisponibility of the drug [73,74]. In other instances, use of CDs result in a slow or sustained release of drugs or in an increase in the rate of dissolution of a solute. These important applications are out of the scope of the review (see for instance [73,75-77]).

Protection against light and oxidation

Extensive research has shown that inclusion of sensitive molecules often results in some kind of protection of the guest molecule from the effect of oxidation and photochemical degradation. Saturated and unsaturated fatty acids (UFA) were the first examples of this protection [78]. There is a masking action against oxygen diffusion and restricted accessibility to the included guest molecule may be involved. These effects are very important for all polyenes such as vitamin A [79], retinoids [50] or diphenylpolyenes [19].

Other applications

Sometimes the inclusion of a guest molecule affords some protection against hydrolysis (phenothiazines, vitamin esters, prostaglandin E). However in chemical applications, the same CDs have been used with other classes of molecules to catalyze various reactions, namely hydrolytic reactions [61,80,81]. In the case of mixed solvents, these reactivity studies show that the organic molecule solvents occupy the cavity of the CD in different ways according to their nature and structure, so that a different space is available for the included analyte [81]. This observation is of interest for the separation of isomers.

Recently, CDs have shown they could behave as efficient chiral selectors and they are used as separating agents in chromatography [82] and capillary electrophoresis. The addition of a complexing borate ion to the CDs improves the selection efficiency [83,84]. A recent review, which offers a complete overview on chiral separation in capillary electrophoresis was recently published by Gübitz *et al.* [85]. It contains a large section on the use of CDs. This is developed further in section 4.4.

Micelles

Structure and properties

Micelles are colloidal aggregates, which are formed by the self-association of monomeric units solubilized in water or in aqueous solutions as well as in organic solvents in the presence of a minimum amount of water. These monomers are called detergents, surfactants, amphiphiles or tensioactive molecules. Their structure is characteristic in that they consist in a long alkyl chain ending with a polar end. The alkyl chains are usually between 8 and 18 carbon atoms long. The polar group or head may be anionic, cationic, zwitterionic or neutral as in polyethers. These detergents form micelles when their concentration reaches a minimum concentration called the critical micellar concentration (CMC). A micelle is an "organized" system by itself as is also its microenvironment. Many of the important and peculiar properties of micelles are a consequence of the self-organization of the monomers. Usually, that is at the lowest concentration where they exist, they are spherical with a radius of about 1 to 3 nm [21,86]. At higher concentration or in special conditions (high ionic strength) they can grow into rod-like shapes [87]. In such experimental conditions the number of monomers can reach very high values and the microviscosity of the medium changes from about 10 to 30 cP and more. If their concentration goes even higher, some of these surfactants may form liquid crystals. The hydrophobic effect, which pushes the alkyl chains to shy away from water, the Van der Waals forces between chains and the electrostatic interactions or capacity to hydrogen bond explain the existence and properties of micelles.

One of the most important characteristics of micelles is their ability to adsorb or solubilize hydrophobic molecules. These molecules which often have a very low solubility in water can be adsorbed onto the micellar surface [88], can move close to the hydrophobic chains of the monomers next to the heads or be buried deeper inside the micelle, in a region called the core [89,90]. These different positions are only average positions since the monomers move around the micelle as well as in and out of the micelle, which is in fact a dynamic structure [91].

One of the most important contribution of fluorescence to the study of micelles is the determination of the mean aggregation number of micelles using a very simple protocole [92]. In order to obtain some information about the localization of solutes in micelles [93], fluorescent probes such as aromatic hydrocarbons [94,95], 1,3-dialkylindoles [96], 7-alkoxycoumarins [97] and a phenantro derivative of quinolizinium chloride [98] have been used. Chemical shifts from NMR studies of pyrene and their polar derivatives such as pyrene sulfonic and pyrene butyric acids give information on the localization of these molecules in micelles [99-101].

Retinal and its 9-*cis* and 13-*cis* isomers all of which are not fluorescent in organic solvents [102,103] emit fluorescence at room temperature in different micellar media (SDS, CTAB, Brij-35 and Triton X-100). The fluorescence intensity is similar to the one obtained in the presence of β-CDs (see 4.2). Retinol, retinyl acetate, retinoic acid (Fig. 4) also exhibit luminescence at room temperature in the same conditions. The emission spectra display well resolved peaks proving that the micellar phase effectively protects the excited states of the retinoids (λ_f = 430 nm for retinal and λ_f = 420-450 nm for retinoic acid) [104,105]. This shows that the solubilization of these polyenes is effective in micellar media and that the protection afforded to the excited states by the different organized media is similar. An exception is retinoic acid for which only a weak emission is obtained in these micellar solutions. Interestingly, when solutions are deoxygenated by addition of sodium sulfite no

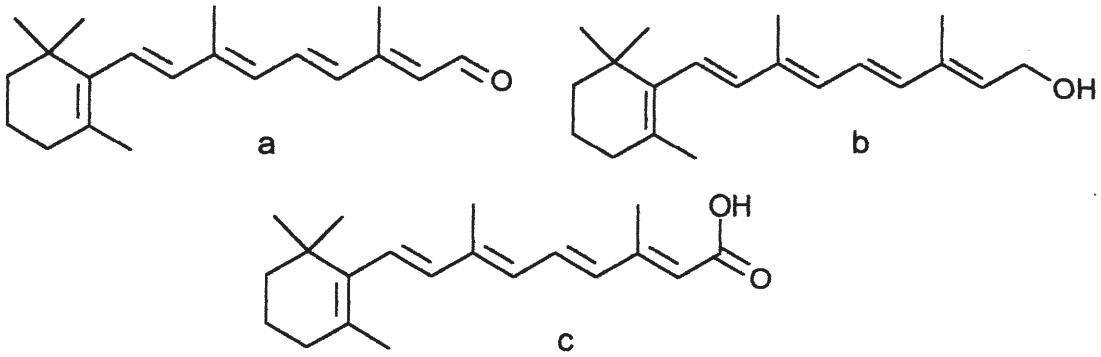

Figure 4. Some retinoids: (a) retinal; (b) retinol (or vitamin A); (c) retinoic acid.

further enhancement of the fluorescence intensity is observed.

Properties leading to applications

Surfactants and micellar solutions are the source of an active field of research as they lead to many applications in chemical and pharmaceutical industries [106,107]. Many of these applications are a consequence of their capacity to solubilize water-insoluble compounds such as vitamins A, D and E, steroids, antibiotics or fatty acids. Besides aqueous surfactant solutions are isotropic, stable, optically transparent and homogeneous. Many compounds exhibit a different reactivity when going from a homogeneous solution to a micellar medium. Reaction rates for many reactions can be easily modified using the appropriate surfactant, and can increase by two or three magnitude orders of magnitude [108]. This behaviour has its origin in the high concentration of analyte and reagents found near the surface of the micelles. Enhancements of the reaction rates have been described for cations and tetraphenylporphyrins [109], chlorophylles [110] and for amines with OPA [111]. Photochemical reactions are also modified in the presence of different micellar media [112-116].

Analytical applications

Advantages resulting from the use of organized media

In the field of analytical applications, the use of cyclodextrins and micellar solutions presents a number of positive features with respect to spectroscopic detection based on absorption or fluorescence: CDs and usual aqueous micellar solutions do not absorb above about 230-240 nm. Furthermore scattering of UV-visible light is generally negligible for these media in the concentration range used for analytical work, so that the determination of hydrophobic molecules is made possible in good conditions. The chemical stability of cyclodextrins is also quite high at pH above 3.5 and for temperatures lower than 60 °C [5].

However, for CDs and specially β-cyclodextrin at higher concentrations, the formation of an extended net of intra- and inter-molecular hydrogen bonds which lower the energy of non-bonding doublets of oxygen results in the formation of aggregates which scatter light more efficiently. For micellar media, an increase in ionic strength may trigger a change in the shape of micelles together with an increase in light scattering.

The positive features referred to above cannot explain alone the extended use of CDs and micellar media. Neither can secondary effects such as the increased stability of an analyte or of one of its derivative after inclusion, one example being the detection of Pd ions in the presence of the (1:1) complex between 1,2-diaminoanthraquinone and β-CD [117].

Two key factors lie at the root of these developments. One is the increase in selectivity during the process of inclusion or solubilization, which may be tuned by modifications brought to the structure or the immediate environment of the CD or by the proper choice of the detergents used to form the micelles. The second is the increase in sensitivity due to an enhancement effect on various emissive properties of the guest molecules following their inclusion or solubilization. The present review will mostly focus on the latter.

Many articles dealing with the use of organized media in classical spectrofluorimetric and spectrophotometric determinations have been published lately [5,118] and will be dealt with in section 4.2. However, during the last decades the most important analytical applications of cyclodextrins and micellar media were focused on separation techniques, mostly HPLC or capillary zone electrophoresis (CZE) and their variants. In particular, the progress in chiral separation have been boosted by the use of cyclodextrins [85,119,120].

Enhancement of the fluorescence of guests molecules in cyclodextrins

It was said before that fluorescent guest molecules in an inclusion complex benefit generally from a better protection from radiative desactivation processes and/or even from photochemical degradation, and generally display an enhanced emission. These two features have been verified for many different compounds and the most promising cases involve drugs and other molecules of biological interest, which have to be analysed in aqueous medium. It is well known that modifications of the electronic properties of the guest start with modifications in its absorption spectrum, as seen for benzene and some of its amino or hydroxy derivatives [65], indole [121] or 1-chloronaphthalene in water or aqueous D-glucose solutions [44]. UV-visible absorption is generally enhanced but at times it may be depressed. Furthermore, the spectrum is usually bathochromically shifted. Emissions in their turn are enhanced and shifted since excited states and ground state molecules interact differently with the CDs [44,65,121,122]. For analytical work, one should pay attention to the fact that the advantage of a modest enhancement may be offset by the fact that the Stokes shift is often reduced for the complexed *versus* the free analyte.

The factors involved in the enhancement are a decrease in radiationless deactivation due to a more rigid microenvironment and restricted available space, a diminished quenching by oxygen compared to organic solvents. Furthermore for molecules quenched by water or transition metal ions, inclusion brings some protection. The enhancement is a positive factor for detection and it may be very high as in the case of dansyl amino-acids, were it varies from 2 to 20 [4,123,124]. These fluorescent probes as well as related molecules are still intensely studied in their interaction with CDs [67]. Recently, the fluorescence enhancement of 1-anilinonaphthalene-8-sulfonate (ANS) by modified β-cyclodextrins was analysed further by Wagner *et al.* (Fig. 5) [125]. Enhancement for these so-called hydrophobic fluorescent probes is mostly due to the fact that the quenching of the emission of the monomer probe by water is prevented by inclusion in the cavity. Naproxen (Fig. 2), a non steroidal anti-inflammatory drug, also contains a substituted naphthalene ring and displays an enhancement of its fluorescence in the presence of CDs. Its limit of solubility in water is very

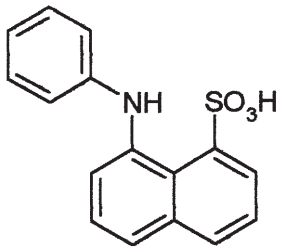

Figure 5. The hydrophobic fluorescent label 1,8-anilinonaphtalene-sulfonate (ANS).

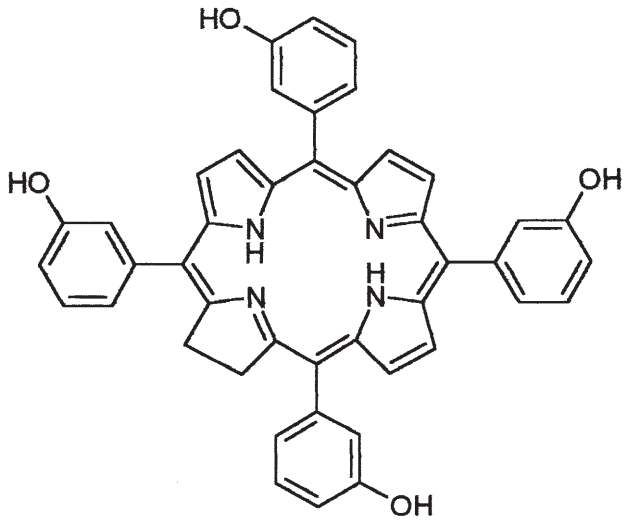

Figure 6. 5,10,15,20-*m*-Tetrahydroxy-mesotetraphenylchlorin (*m*-THPC), a sensitizer for photodynamic therapy.

low and until recently was not agreed upon. Recently, Junquera *et al.* have determined this value (3×10^{-5} M) and shown that the choice of the pH of the solution was very important due to the presence of an acetic acid substituent. The neutral and acid forms of the molecule are present and form different complexes with hydroxypropyl β-cyclodextrin [126]. Naproxen is used in various pharmaceutical formulations, which contain hydrophilic molecules. It complexes with the latter to offer an overall improved solubility and bioavailability. An analysis of naproxen based on fluorescence must take into account the fact that these interactions may affect its emission. For instance, it has been shown by Vélaz *et al.* that vinylpyrrolidone quenches the fluorescence of naproxen [127]. At pH values for which the neutral form dominates however, the binding to the CD is higher and fluorescence is enhanced. Another example of a strong effect of pH on inclusion was described for salicylic acid-salicylate system in the presence of hydroxypropyl-β-cyclodextrin. The pH dependent fluorescence enhancement is analyzed in aqueous solutions at 25 °C [128].

A strong fluorescence enhancement is also observed for 5,10,15,20-tetra-(m-hydroxyphenyl)chlorin (*m*-THPC, Fig. 6), a sensitizer used in photodynamic therapy (PDT). The emission intensity following its complexation with natural and modified cyclodextrins is up to 300 times more intense whereas the molecular absorption coefficient is hardly two times higher than for the cyclodextrin free solution [129]. The explanation lies in the fact that in the (1:2) complex, the phenolic groups interact strongly with the cyclodextrins which encapsulate the guest. The low yield in the absence of cyclodextrins may be due to the easy formation of non-emitting oligomers of porphyrins or to quenching following charge transfer or deprotonation of the phenolic groups in

the excited state. In the same field of PDT, Mosinger prepared a soluble supramolecular sensitizer by complexation of meso-tetrakis(4-sulfonatophenyl)porphyrin with 2-hydroxypropyl-cyclodextrins [130].

Phenothiazines constitute an important class of drugs, mostly antidepressors, which have in common a strong sensitivity towards photodegradation since they are colored compounds. Several recent studies relate the fluorescence properties of phenothiazines complexed to CDs in aqueous media. The photoprotection offered to promazine in aqueous solution in the presence of CDs has been analysed by Lutka [6]. Maafi *et al.* looked at a 2-hydroxypropyl-β-cyclodextrin:benzo[a]phenothiazine inclusion complex and noted a 12-fold increase in fluorescence intensity relative to the free analyte [131,132]. The authors have used this feature to develop the first spectrofluorimetric determination of this anticancerous drug in the presence of cyclodextrins. In their experimental conditions 97 % of the analyte is complexed for a 0.03 M concentration in HP-β-CD. The detection limit of 7 $\mu g\ l^{-1}$ is a proof of the analytical usefulness of the formation of this (1:1) complex. The same group used the photochemically induced fluorescence of another phenothiazine, Azure A, in the presence of β-CD to further enhance the fluorescence of the photoproduct of Azure A. The native fluorescence of this positively charged phenothiazine is very weak. UV irradiation of a solution of Azure A forms a neutral sulphoxide which has a 30-fold more intense fluorescence. Adding the CD to the UV irradiated solution results in another 3-fold intensification of the fluorescence accompanied by a 20 nm blue-shift of the emission [133]. The photochemically induced fluorescence has been used also for the determination of different classes of compounds such as aromatic pesticides. The ones which are non emitting form fluorescent (1:1) complexes in aqueous solution after irradiation and all show an enhancement (3 to 12-fold) of fluorescence in the presence of various cyclodextrins [134]. The analytical figures of merit have been evaluated and the results show that a determination for water from rivers seems possible.

Compounds derived from carbazole and carbazole itself [135] have been widely studied in the past years. Ellipticine, the most representative of the pyrido[4,3-b]carbazole alkaloids (Fig. 7) exhibits a strong antitumour activity. But the use of this neutral form is hampered by its extremely low solubility in water and associated detection problems in clinical analysis. So it is administered as a salt; however, nitrogen quaternization modifies the anti-tumour properties of ellipticine. Potential alternatives to quaternization include the use of cyclodextrins or the use of micellar media. An inclusion complexe between γ-CD and ellipticine was characterized by El Hage Chahine *et al.* after a very thorough study [136]. A (1:2) complex involving the neutral form is described; however the ellipticinium form of the drug is not complexed. Recently, the stoichiometry and association constants were obtained for inclusion complexes between ellipticine and β-CD and for a set of complexes with modified β-cyclodextrins, including hydroxypropyl-β-cyclodextrin (HPβ-CD), (2,6-di-O-methyl)-β-cyclodextrin (DM-β-CD) and (2,3,6-tri-O-methyl)-β-CD (TM-β-CD as well as with γ-CD [29,30,31,135,137]. The stoichiometry of these complexes was calculated on the basis of spectrophotometric (UV-Vis) and spectrofluorimetric measurements. The stoichoimetry was 1:1 (Elli:CD) in the case of the complexes with β-CD and modified β-CDs. Association constants were calculated using spectrofluorimetric data obtained while keeping ellipticine concentration constant at 1.0×10^{-6} M and varying the cyclodextrin concentrations. Here also, the association constants depend on the pH of the medium and therefore they were calculated at different pH values (1.0, 9.2 and 13.0) for which different forms exist for ellipticine (neutral, zwitterionic, cationic and anionic). These forms may coexist in equilibria depending on the pH and the solvents. Modified β-CD (DM-β-CD, and HP-β-CD) together with γ-CD gave the higher association constants.

Other studies on fluorescence enhancement have been carried out with coumarin derivatives [138,139]. In the latter the authors describe a rapid determination of warfarin, a coumarin derivative (Fig. 8) used a rodenticide or as an antithrombotic drug. They use a sequential injection analysis method with a 0.02 $\mu g\ ml^{-1}$ detection limit requiring 20 s for one analysis. Although warfarin is itself strongly fluorescent, it was shown by Ishiwata *et al.* that CDs were enhancers of the fluorescence, with β-CD performing best [140]. In the procedure set-up by Tang *et al.*, its signal could be amplified about 3.5 times. The natural flavonoids rutin and hesperidin (Fig. 8) have a chromophore very similar to that of a coumarin (substituted benzo-4-pyrone instead of benzo-2-pyrone). Rutin shows an enhanced fluorescence by complexation with various CDs [141-143]. Hesperidin, which is extracted from the peel of oranges, acts as an efficient anti-oxidant and possesses anti-viral properties. In the presence of HP-β-CD and M-β-CD (β-CD with a 1.8 degree

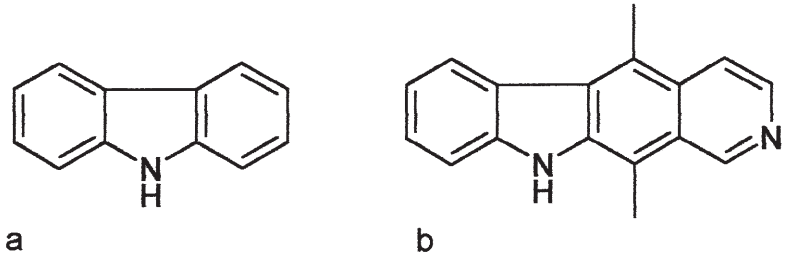

Figure 7. (a) carbazole; (b) ellipticin.

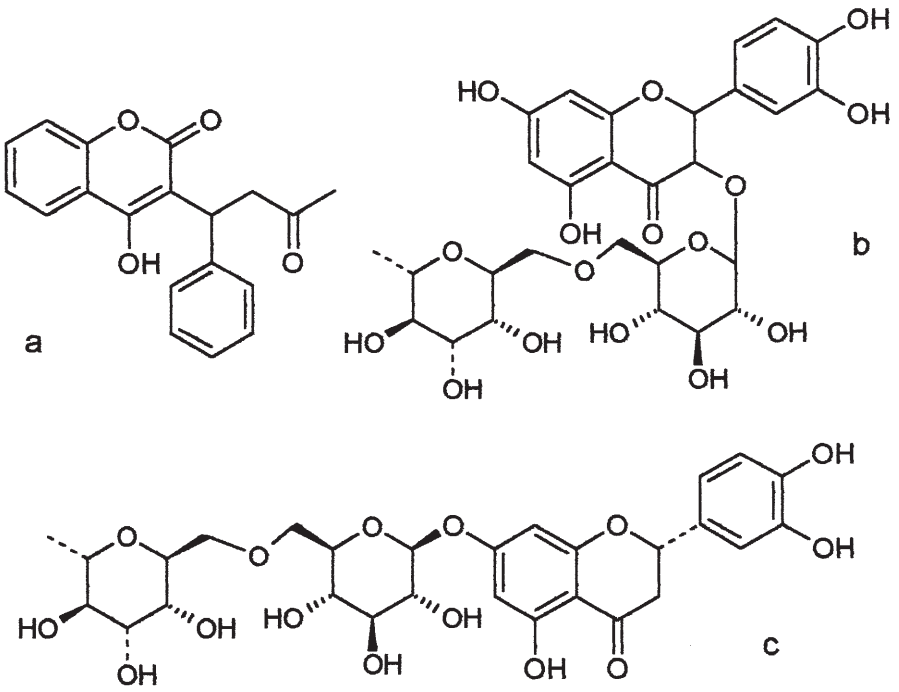

Figure 8. Derivatives of coumarin and analogues: (a) warfarin; (b) rutin; (c) hesperidine.

of substitution by methyl groups) its fluorescence is enhanced 17 and 26 times respectively by formation of a (1:1) complex. The better protection offered by M-β-CD is confirmed by the lowest Stern-Volmer constant obtained in quenching experiments by bromide ions for all CDs tested [144].

Another publication reports on the enhancement of analogous molecules from a physicochemical point of view. A study on aminocoumarins [145] emphasizes the quality of the specific interaction with CDs to obtain a strongly enhanced fluorescence and stabilize the included coumarins. Their aim is to obtain better lasing conditions for these dyes.

Much work was also carried out on molecules belonging to the polyenes family. Retinol and its derivatives, the so-called retinoids, are of interest for their properties related to the membranes of cells, to cell proliferation and differentiation [73] or as supplement in the diet [146]. Retinal, which plays also a major role in the process of vision, belongs to this family. Retinoids are very sensitive to oxidation or isomerization under the effect of light or the combined effect of light and oxygen [103,147,148]. Furthermore, they are generally insoluble in water. As a consequence, they are difficult to quantify in aqueous media such as biological media. Earlier studies with CDs have shown that their inclusion in cyclodextrins increased their stability [1,76,79] or their solubilization in aqueous solution [149]. This was also shown for retinoic acid [74] and for retinal which has a very low solubility in water ($< 10^{-7}$ M) [149]. However, these studies were not based on the monitoring of emissive properties. Retinol and its esters are weakly fluorescent in organic solvents at room temperature [102,103]. Retinoic acid and retinal emit even more weakly at room temperature in carefully de-oxygenated organic solution [102,103] and it appeared that complexation with CDs should enhance those emissions. The first study to report on the systematic effect of CDs on the luminescence of retinoids appeared in 1989 [104]. The key result is that even retinal could give off a measurable luminescence at room temperature when complexed to various CDs. The specificity of the cavity favours the complexation with β-CD, α-CD being too small and γ-CD too large to provide a good fit or a tight protection respectively. The hope that such complexed retinoids would lend themselves to a better quantification was then curbed by the difficulties met with retinoids and a methodology was first developed with the analogous molecule 1,6-diphenyl-1,3,5 hexatriene (DPH, Fig. 9) to follow the formation of the complexes. DPH is insoluble in cold water and its complexes were detected by enhancement of fluorescence on TLC plates. Free DPH and the inclusion complex are so well separated that the quantification of the unbound molecule is

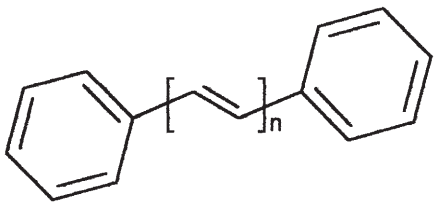

Figure 9. The diphenylpolyenes: DPH corresponds to *n* = 3.

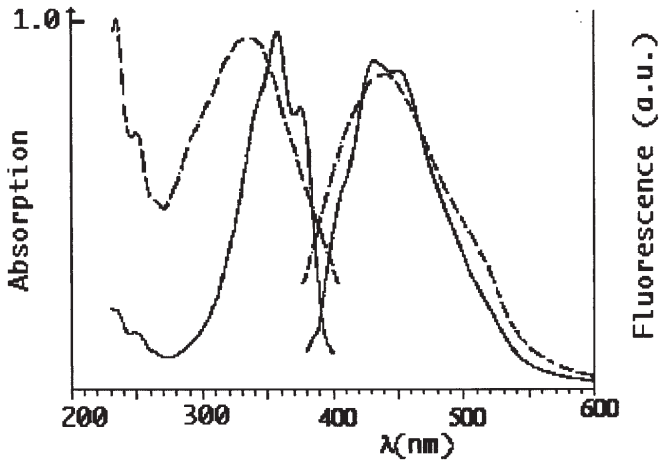

Figure 10. Absorption and emission of a complex of retinal (––––) and a complex of retinoic acid (-----), both with β-CD in water.

carried out without interference by means of a CCD camera [18]. Differences in the absorption spectra of the free and included DPH are taken into account to determine the quantity of included DPH. Simple kinetic studies show that the oxidation and isomerization of DPH are decreased in the inclusion complex [19]. Further studies on the complexation of retinal have then shown that retinal and most of its isomers, as well as retinoic acid, retinol and retinyle acetate (Fig. 4) all emit a room temperature luminescence when they are included in the following CDs: α-CD, β-CD, γ-CD, DM-β-CD, TM-β-CD and HP-β-CD [50,105]. It is also observed that the fluorescence of retinol is strongly enhanced in the presence of surfactants or cyclodextrins as it is in organic solvents at low temperature [103,150]. The more intense fluorescence is developed for the complexes of the different retinoids with β-CD or HP-β-CD and the least intense with α-CD and TM-β-CD (Fig. 10). This behaviour can be explained as follows: the hydrophobic part of the retinoids are the trimethyl-cyclohexenyl ring and the polyenic chain and it is expected that either part of the molecule could be in contact with the CDs cavity. As α-CD has a small cavity and the methyl groups of TM-β-CD present an important steric hindrance, even partial inclusion is difficult and the protection afforded by these CDs is reduced (Fig. 11). The 13-*cis*- and 9-*cis*- isomers of retinal were also studied and it is possible to distinguish the all-*trans*, 13-cis and 9-*cis*-isomers on the basis of the different fluorescence emission intensity and shifts with the same type of cyclodextrin. The lowest fluorescence intensity is obtained for the 9-*cis* isomer. These results allow the selective and quantitative determination of retinoids in an aqueous medium.

Metal ions can also be complexed by cylodextrins. Enhancement was observed for a ternary complex involving the metal ion terbium, trimesic acid and a CD, when a zirconate salt is added. This effect was used to develop a determination of terbium [151]. An original bioanalytical technique for the determination of glutamate and analogs was reported by Santra *et al.* A fluorescent (1:2) pyrene β-CD complex is quenched when Cu(II) ions are added to the solution. The total quenching of fluorescence results from the binding interaction of two copper ions with the (1:2) complex which are held close to pyrene so that a (1:2:2) complex is formed. When glutamate is added, it takes the Cu(II) ions off the ternary complex since the affinity of glutamate for the copper ions is stronger than the affinity of the copper ion for the (1:2) complex, and the pyrene is no more quenched [152]. Ueno *et al.* prepared a set of CDs substituted by chromophores sensitive to the complexation of organic molecules [38,39] and to the complexation of a metal ion [153]. The latter is substituted by both, monensin a polyether able to complex metal ions and N-alpha-dansyl-L-lysine. The fluorescence of the latter is affected by the

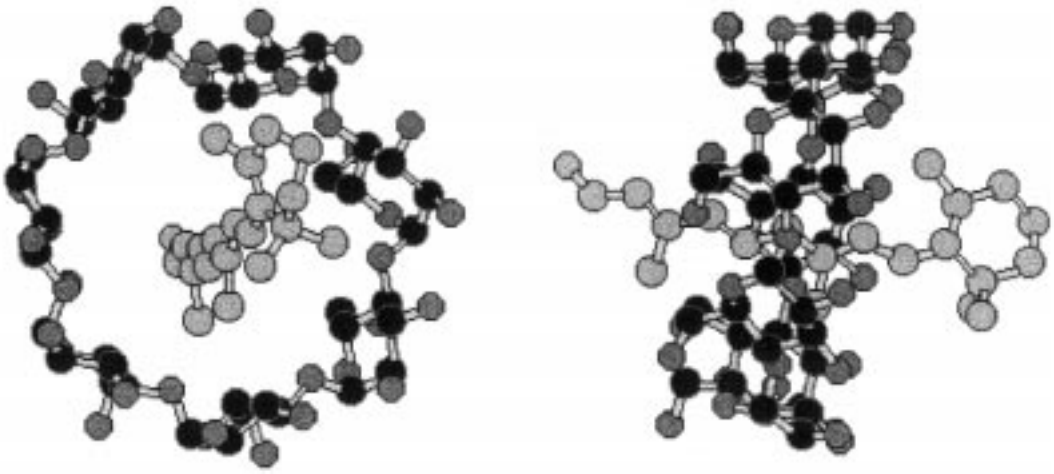

Figure 11. Modelisation of a complex of all-trans retinal with β-cyclodextrin (using Hyperchem v. 6.0 from Hypercube). In such a complex, the major contribution to the energy of the complex comes from Van der Waals interactions between the side chain of retinal and the cavity of the CD.

binding of the ion. Another typical analytical application of the enhancement effect can be seen in an attempt to replace horseradish peroxidase (HRP) in the spectrofluorimetric determination of H_2O_2. An α-CD-hemin complex was studied as a replacement for HRP. A pH of 10.4 proved to be the optimum for the experiment and ammonia was found to enhance the peroxidase activity of the α-CD-hemin complex. The oxidation product 2,2'-dihydroxy-4 4'-dimethylbiphenyl was monitored fluorimetrically and a detection limit of 3.4×10^{-9} mol l^{-1} was reached for H_2O_2 [154].

A fiber optics fluorescence based sensor was described for riboflavin. It is based on the fluorescence enhancement of an evanescent-wave excited beta-cyclodextrin complex trapped in a sol-gel-derived porous coating [155].

Solubilisation and analysis of fluorescent analytes in micellar media

The association of micellar media and luminescence for the purpose of carrying an analysis is not recent and produced a large volume of publications [10,156,157]. This is true for direct micelles and for microemulsions or reversed micelles as well [158,159]. Procedures based on fluorescent enhancement were defined for simple determination such as that of pyrene [160] and aminoacids [161] or for complex samples like coal liquids. In the latter example, the efficiency of bile salt and conventional detergent micelles were compared [162]. Micellar media were also probed as an analytical tool for the fluorimetric determination of ellipticine [137] using anionic (SDS), cationic (CTAB) and neutral (Brij-35] surfactants. Fluorimetric analysis shows that ellipticine is totally solubilized. The slope of the calibration curves are considerably improved as are the correlation coefficients (*e.g.* 0.8904 in water and 0.9982 with SDS). Micellar media also modify proton transfer processes: deprotonation of ellipticine is hampered in anionic SDS due to the surface charge of the micelles. Finally, fluorescence of the solubilized ellipticine is protected from quenching by bromide ion.

Lanthanide ions, acting as counter ions, were used as efficient energy transfer agent to detect organic analytes in reverse micelles [163]. The analysis of metal ions also benefit from an enhanced luminescence. For instance, Garcia de Torres *et al.* describe a spectrofluorimetric determination of zinc at ultra-trace levels [164] and an increase in the sensitivity for the detection of aluminium was observed after complexation with morin in the presence of surfactants [165].

Recently, studies appeared where cyclodextrins and surfactants were mixed. The results show that there is an interaction between both components in absence of a potential analyte, with the result that the CMC is shifted towards higher concentration in surfactant. This was monitored in the case of Triton X-100 with various cyclodextrins using the fluorescence of toluidino-naphthalene sulphonate (TNS) as a probe [166]. At 10 mM β-CD the CMC is increased 28 times whereas it is not affected by the presence of α-CD. A competitive binding occurs between the probe and the surfactant. Similarly, the effect of added β-CD on the micellization of cetyltrimethylammonium bromide (CTAB) in aqueous solution was probed by the dual fluorescence of sodium p-dimethylaminobenzoate [167]. The same upward shift is noted for the CMC.

Micelles and cyclodextrins in separation science using fluorescence detection

The applications of luminescence spectrometry in HPLC are numerous and have been reviewed on a regular basis [168]. The use of micelles as an enhancing means in chromatography is not new either [169] but it has seen a strong extension in the recent years. A few recent examples are given hereunder.

A determination of steroids in urine by micellar HPLC with detection by sensitized terbium fluorescence was given by Amin *et al.* [170]. Sensitized lanthanide luminescence was also used for the determination of theophylline by liquid chromatography [171]. It involves triplet energy transfer from the analyte to the Tb ion in the presence of micelles.

Micelles are frequently used in micellar electrokinetic chromatography (MEKC) as a pseudostationary phase to increase the efficiency and the resolution of the capillary electrophoresis separations. They offer new possibilities for the determination of neutral compounds by capillary electrophoresis, specially in the case of drugs and pharmaceuticals excipients [172]. The influence of the surfactants on the migration of analytes in capillary electrophoresis has been established [173] and the theoretical migration modes and their application to the determination of metal complexes has been described [174].

A mixture of alkaloids deriving from crude morphine, poppy straw or opium preparations is successfully separated by micellar electrokinetic capillary electrophoresis (MEKC) using cetyltrimethylammonium bromide in the working buffer [175]. The results are in agreement with those obtained using HPLC, and similar coefficients of variations are obtained. In a similar work, Björnsdottir and Hansen [176] determine the five major alkaloid components of opium in opium and its drug formulations. These authors use the benefit of host-guest complexation obtained by addition of a surfactant (Tween 20) and cyclodextrins to develop a validated approach.

Cyclodextrins, grafted or polymerized, can be used to provide a host-guest mediated separation of complex mixtures or as chiral stationary phases to separate enantiomers.

A comparative study of fluorescence enhancement was published by Jin *et al.* for systems using different ß-cyclodextrin derivatives and cyclodextrin-surfactant media [177]. Fluorescence enhancement was used in the high performance size exclusion chromatographic determination of sulphobutylether-β-cyclodextrin in human plasma [178]. It proved to be a very selective method. HPLC with fluorescence detection is used also to obtain the formation constants of analytes with the cyclodextrins added to the mobile phase. Two recent studies use the technique, one on pyrene and anthracene [71] and one on a set of new derivatization agents mostly derived from carbazole [179].

Recently, Smith *et al.* provided the first separation of metabolites of PAHs using γ-CD and MEKC [180]. They obtain baseline separation of 12 hydroxyPAHs with a laser-induced fluorescence (LIF) detection. Cyclodextrin-aided capillary electrophoretic separation and LIF detection were also used to determine mixtures of the 16 US EPA priority pollutants PAHs from contaminated soils [181,182] and the results are comparable to those afforded by HPLC. The indirect fluorescence detection of amino-substituted PAHs in CD-modified MEKC was demonstrated. The fluorescence intensity of the probe oxazine 750 increased in the presence of γ-CD following the formation of an inclusion complex. Addition of the amino-PAHs decreases the signal (which is excited by a diode laser). The results obtained agree with a proposed model of interaction between analyte and the probe [183].

In a different field of application, a separation following derivatization was performed by cyclodextrin-modified capillary electrophoresis with LIF for resin acids (RAs) commonly found in untreated pulp mill effluents. The procedure involves the reaction of 4-bromomethyl-7-methoxycoumarin a fluorescent label for the carboxyl group of the resin acid. The derivatized esters are very stable, emitting fluorescence at 400 nm following excitation at 325 nm. The cyclodextrin-modified capillary electrophoresis uses a mixture of negatively charged sulfobutylether-β-CD and neutral methyl-ß-CD. Baseline separation of eight very closely related derivatized resin acids is obtained, the limit of detection (3σ) being close to 15 µg/l [184].

The chiral recognition mechanism of β-CD was analyzed by Li and Purdy [5] and modelization studies gave support to an enantioselectivity afforded by chemisorbed β-cyclodextrins [185] or polymeric surfactants [186]. Cyclodextrins have been used to increase the resolution in capillary electrophoresis of anti-inflamatory drugs of the profen family [187] of propanolol [188] and other important drugs such as opiates and fenfluramine enantiomers [176,189,190]. They have also been successfully employed as chiral stationary phases in HPLC for trimetoquinol, denopamine and timepidium [191,192] or profens [193]. Stefansson and Novotny introduced boric acid to improve the electrophoretic resolution of monosaccharide enantiomers. The formation of a borate-oligosaccharide complexation medium improved separation monitored by LIF of a derivative of naphthalene [83]. A similar study was carried out on the capillary electrophoretic chiral resolution of vicinal diols by complexation with borate and CD [84]. Baseline separation was obtained with four diols.

Conclusion

The results reviewed show that all organized media in which CDs or micelles are involved increase the luminescence intensity of fluorescent analytes or probes. Complexation by cyclodextrins or compartimentalization in micelles may even induce the emission of molecules such as retinoids which normally do not exhibit any luminescence in homogeneous solution at room temperature, due to very effective radiationless deactivation processes. In these conditions the protection of excited states can be very effective. However inclusion in cyclodextrins on the average allows a stronger enhancement of the fluorescence than micellar solubilization. For separation techniques, the combination of both classes of molecules, CDs and surfactants, results in a wider array of separation, in particular for neutral molecules. CDs however are necessary for the separation of enantiomers. This field of research is still wide open and new classes of molecules are expected to gain importance in this respect. Calixarenes for instance, which have been the subject of much synthetic developments, are making inroads into analytical and separation techniques. They will be useful not only for fluorescence based detection schemes for metal ions but for organics such as amines for instance [194].

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