Chapter 19

Cyclodextrin Based Spectral Changes

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Additional information is available at the end of the chapter

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1. Introduction

1.1. Cyclodextrins

A cyclodextrin (CyD) is a cyclic oligomer of α-D-glucose formed by the action of certain enzymes, Bacillus amylobacter, on starch. The first reported reference to a cyclodextrin was published by Villiers in 1891 [1]. Three cyclodextrins are readily available: α-CyD, β-CyD and γ-CyD having six, seven and eight glucose units respectively. They are commonly referred to as the native CyDs. For a long time, only the three parent CyDs were known, but during the past decade many covalently modified CyDs have been prepared from the native forms [2].

The glucose units are connected through glycosidic α-1,4 bonds. As a consequence of the \(^{4}C_1\) conformation of the glucopyranose units, all secondary hydroxyl groups are situated on one of the two edges of the ring, whereas all the primary ones are placed on the other edge. The ring, in reality, is a conical cylinder, which is frequently characterized as a doughnut or wreath-shaped truncated cone. It is, of course, the possession of this cavity that makes the CyDs attractive subjects for study. The most notable feature of cyclodextrins is their ability to form inclusion complexes (host–guest complexes) with a very wide range of solid, liquid and gaseous compounds. Complex formation is a dimensional fit between host cavity and guest molecule [3]. This phenomenon bears the name molecular recognition [4].

1.2. Inclusion complex formation

The lipophilic cavity of cyclodextrin molecules provides a microenvironment into which appropriately sized non-polar moieties can enter to form inclusion complexes [5]. No covalent bonds are broken or formed during formation of the inclusion complex [6]. The first driving force of complex formation is release of enthalpy-rich water molecules from the cavity. The second critical factor is the thermodynamic interactions between the different components of
the system (cyclodextrin, guest, solvent). The cavity size of the toroidally shaped CyDs and the structural conformation and size of the guest molecule are the other parameters that mostly affect the formation of a guest-CyD complex [2]. As the results of this inclusion, changes of the chemical or physical properties of both host and guest molecules are generally observed; opening a wide field of applications in many areas and allowing one to monitor the process by several experimental techniques [2,7-9].

![Figure 1. Structure of α-CyD, β-CyD and γ-CyD](image)

**2. Results**

**2.1. Cyclodextrin based spectral changes**

As the result of inclusion complexes formation, the guest molecule is surrounded by the hydrophobic microenvironment of the CyD cavity. This environmental changes cause to some considerable changes in chemical properties of guest molecule such as equilibria and kinetic parameters and some changes in physical properties such as absorption coefficient or quantum yield, these changes strongly depend on the difference between CyD cavity and the outer medium.

Spectroscopic techniques are the most frequent ones which have been used for the study of these changes. Although it should be noted that the phase-solubility is one of the simplest techniques which have been used other than spectroscopy [10].
Some of the spectroscopic techniques such as UV-Visible, fluorescence, and NMR spectroscopy are compatible for the spectral study of the complexes that obtained in solution [11]. But the infrared spectroscopy, X-ray diffraction, scanning electron microscopy techniques [12,13] and differential scanning calorimetry [14], are suitable for the inclusion compounds that obtained in the solid state.

Figure 2. NMR spectra of the trans-1,4-bis[(4-pyridyl)ethenyl]benzene (BPEB) bridged ligand as function of time for the self-assembling ([Fe(CN)₆]₂(BPEB-β-CyD))₆⁻ rotaxane, upon addition of 2 equivalents of β-CyD to the dimer in D₂O: (a) 0 min, (b) 5 min, (c) 30 min, (d) 60 min and (e) 24 hours.
Among the above techniques some of them such as X-ray diffraction and NMR are proper for obtaining qualitative information about the inclusion complex. For example $^1$H NMR spectra can give us some information about the host to guest mole ratios and stability constant and even the orientation of the guest in the host cavity in solution which no other technique can give.

This section provides a condensed overview of the quantitative applications of host-guest interactions and molecular recognition which are well-matched with more quantitative techniques such as UV-Vis absorption and fluorescence.

### 2.2. UV. Vis. Spectral changes

In spite of the small effects encountered in absorption, peak shifts of the order of a few nm and changes of the absorption coefficients less than ten percent, UV-Vis spectrometry is an easily performed first test of the occurrence of complexation in particular in nonfluorescing systems. Moreover, the power of modern chemometric techniques allows valuable analytical applications of small effects of CyD inclusion on UV-Vis spectra. The emphasis of absorption changes and absorption studies will be on the apparent changes in the chemical properties of guest molecules, such as acid–base equilibrium. The most distinguished work in this field is report by Taguchi [15]. He has demonstrated that upon the binding of phenolphthalein to $\beta$-CyD cavity in aqueous solution at pH 10.5, the red-colored dianion form is rapidly transformed into a colorless lactonoid form.

![Figure 3. Proposed mechanism for the colour change of phenolphthalein in the presence of $\beta$-CyD.](image)

This effect and some other similar spectral changes may reflect the altered polarity of the cavity microenvironment and preferential or specific guest–host interactions and stabilization of the preferred form and suppression of the other form in equilibrium. This is not a comprehensive review but is mainly intended to provide illustrative examples.

The absorption spectrum of mycophenolate mofetil (MMF) at mild acidic solutions shows an absorption band which has an absorption maximum at 302 nm for its acidic form (HMF). With
the increasing of pH, the absorption at 302 nm gradually decreased whereas the absorption with the 340 nm maximum, for the basic (MF\(^{-}\)) form, increased, Fig. 4. These spectral changes and presence of an isobestic point indicate the presence of acid base equilibrium for this immunosuppressant drug.

The spectra of MMF in the presence of varying amounts of \(\beta\)-CyD at constant pH that both acidic and basic forms are presented in solution are shown in Fig. 5. The spectral change by increasing the \(\beta\)-CyD concentrations at constant pH is similar to the decreasing the pH of aqueous MMF solution. These spectral changes indicate suppression of the basic form and dominance of acidic form in the presence of \(\beta\)-CyD cavity.

Figure 4. The absorption spectra for \(4.0\times10^{-4}\) mol L\(^{-1}\)MMF at various pH values. The pH values are (a) 5.0, (b) 6.5, (c) 7.0, (d) 7.5, (e) 8.0, (f) 8.5, (g) 9.0 and (h) 9.5. [Reprinted from Khalafi L, Rafiee M, Mahdiun F, Sedaghat S. / Spectrochim. Acta Part A., 2012; 90 45-49 with permission from Elsevier Science.]
Rank Annihilation Factor Analysis (RAFA) is used as an efficient chemometrics algorithm for the analysis of spectrophotometric data and the conditional acidity constant of MMF and the stability constant of its acidic and basic forms were obtained in the absence and presence of β-CyD. Based on these results with increasing β-CyD concentration the acidic form stabilized and the equilibrium of the system driving to produce acidic form. Consequently the conditional acidity constant decrease with increasing the β-CyD concentration [16]. The spectrophotometric study of neutral red and 4-nitrophenol in the presence of β-CyD are the other examples of spectral changes with different preferential complexation.

In the case of neutral red the increase in the acidity constants as a function of β-CyD is indicative of more stabilization of basic (neutral) form rather than positively charged acidic form. Whereas the study of acid-base equilibrium of 4-nitrophenol show that 4-nitrophenolate (the negatively charged basic form) has more affinity than the acidic (neutral) form. It has been claimed that the driving force of more stable inclusion complex of 4-nitrophenolate with β-CyD is the hydrogen bonding [17, 18].

The above results and some other comprehensive studies show the effect of interaction of guest molecules with microenvironment of β-CyD cavity. The CyD nanocavity has the characters similar to an 80% dioxane/water solution and provides a slightly alkaline environment [19]. There are four possible interactions including; hydrophobic, hydrogen binding, Van der Waals forces and donor-acceptor for the cavity that affect the favored interaction, equilibrium shift and spectral changes in the presence of β-CyD [20-22].
2.3. UV. Vis. based Molecular recognition:

The spectral change of an indicator may not be important in molecular recognition itself, but there is an important concept named as “indicator displacement assay” and/or “spectral displacement” which have been developed considering these spectral changes. Spectral displacement method involves the color changes upon addition of competitive guest molecules; the dye moiety was excluded from the CyD cavity and located in the aqueous media. In that state, by environmental changes around the dye moiety, the dye moiety shows its normal color changes resulting from pH changes [23].

![Suppression of Color Change](image1)

![Normal Color Change](image2)

**Figure 6.** p-Methyl red appended β-CyD chemical sensor

A spectroscopic displacement method is used to determine association constants or the concentrations of the compounds that are spectroscopically transparent. Each application may be divided into two classes, the first one is based on competitive inclusion of guest and indicator in the solution, and the second one is the competition of dissolved guest with the CyD bonded indicators.

The success of the visible spectral displacement technique involving methyl red, in bonded form, as the competing reagent applied for the construction of molecular sensor for adamantancarboxylic acid, adamantanol, borneol, cyclaxtanol, cyclohexanol and same structures [24, 25].
The spectrophotometric technique involving phenolphthalein as the competing reagent appears to be the most promising one. It is based on the fact that in alkaline solutions a colourless 1:1 complex is formed between phenolphthalein and β-CyD that the red phenolphthalein dianion is partially displaced by a competing reagent to an extent depending upon its affinity to form a complex with the CyD host. Phenolphthalein-modified β-CyD was synthesized for the purpose of developing a new type of guest-responsive color change indicator and the guest-induced absorption changes were used for molecule sensing [26, 27].

Figure 7. Absorption spectra for $4.8 \times 10^{-5}$ mol L$^{-1}$ phenolphthalein in the presence (a) 0.0, (b) $1.0 \times 10^{-4}$, (c) $2 \times 10^{-4}$, (d) $4 \times 10^{-4}$, (e) $7 \times 10^{-4}$, and (f) $1.0 \times 10^{-3}$ mol L$^{-1}$ of β-CyD at pH 10.5. [Reprinted from Afkhami A, Madrakian T, Khalafi L. / Anal. Lett, 2007; 40 2317-2328 with permission from Taylor & Francis.]

Several attempts have been also made on color changes based on competitive complexation of some important chemicals with phenolphthalein-CyD inclusion complex. These chemical sensors are relatively inexpensive, rapid and simple for determination of desired compounds, such as pharmaceuticals, surfactants and fatty acids which are transparent in the visible range [28-34]. The sensing abilities of various guests are roughly parallel to the binding constants. Fig. 8 shows that by addition of ibuprofen to the phenolphthalein-β-CyD complex solution, the absorbance at 554 nm increases. This increase in the absorbance is due to the decomposition of phenolphthalein-β-CyD inclusion complex by displacement of ibuprofen by phenolphthalein. This phenomenon indicates competition of the ibuprofen with phenolphthalein in the formation of inclusion complex.
with β-CyD. The amount of increase in the absorbance at 554 nm was found to be proportional with the ibuprofen concentration over a certain concentration range.

**Figure 8.** Absorption spectra for 4.8 × 10⁻⁵ mol L⁻¹ phenolphthalein at pH 10.5 in the presence of (a) 1.0 × 10⁻⁴ mol L⁻¹ β-CyD and 2.0 × 10⁻⁴ mol L⁻¹ ibuprofen, (b) 1.0 × 10⁻⁴ mol L⁻¹ β-CyD and (c) in the absence of β-CyD and ibuprofen. [Reprinted from Afkhami A, Madrakian T, Khalafi L. /Anal. Lett, 2007; 40 2317-2328 with permission from Taylor & Francis.]

Color change chemical sensors of CyD derivatives carrying dyes such as nitrophenol [35] and alizarin yellow [36] were reported that relies on direct measurements of some analytes.

Also there is an example of color and spectral change of metal ion-indicators that affected by β-CyD. Recently it has been demonstrated that the addition of β-CyD to the solution containing the complex of calcium and magnesium with Eriochrome Black T (EBT) caused decomposition of the 1:1 metal complex and increase in EBT concentration in solution due to the formation of EBT-β-CyD inclusion complex. At a given pH, the values of metal ion conditional formation constant (K'f) decreased by increasing β-CyD concentration based due to the formation of an inclusion complex between the desired form of EBT and β-CyD. The amount of decrease in K'f with increasing β-CyD concentration and
the color changes due to complex decomposition depends on the stability of the inclusion complex between EBT and β-CyD [37].

There is a large volume of published studies reporting the affinities and even selective affinity of secondary hydroxyl side of CyDs for metal ion binding and complexation [38]. This complexation ability improves considerably by structural and functional groups modification. The secondary hydroxyl groups are deprotonated and coordinated to bind Pb(II) ions forming a hexadecanuclear lead(II) alkoxide [39]. Two amino groups introduced on the primary hydroxyl side of β-CyD can chelate a platinum ion [40]. 6-amino-glucopyranose analogue of β-CyD had binding affinity for metal ions with Cs⁺ selectivity [41]. In 2010, Pitchumani et al. reported a per-6-amino-β-CyD as a supramolecular host and p-nitrophenol as a spectroscopic probe as a novel colorimetric and ratiometric sensor for transition metal cations, Fe³⁺ and Ru³⁺ in water. Binding of these cations causes an appreciable change in the visible region of the spectrum which can be detected by naked-eye and is insensitive to other metal ions namely Ag⁺, Cu⁺, Mn²⁺, Fe²⁺, Cu²⁺, Zn²⁺, Cd²⁺, Hg²⁺, Pb²⁺, Cr³⁺, La³⁺ and Eu³⁺. The color change and consequent sensing ability is significant at equimolar ratio of host and guest and also at very low concentration [42].

Figure 9. The spectra of Ca-EBT complex (1.0 × 10⁻³ mol L⁻¹ Ca²⁺ and 4.0 × 10⁻⁵ mol L⁻¹ EBT) in the presence of (a) 0.0, (b) 3.0 × 10⁻³, (c) 6.0 × 10⁻³, (d) 9.0 × 10⁻³, (e) 1.2 × 10⁻² and (f) 1.5 × 10⁻² mol L⁻¹ of β-CyD at pH 9.5. [Reprinted from Afkhami A, Khalafi L. / Supramol. Chem., 2008; 19 579-586 with permission from Taylor & Francis.]
Numerous studies have attempted to explain the possibility of incorporation of CyDs and modified CyDs in the structures of ternary complexes as ligand. In some of them the whole complex act as a guest and the metal ion has no direct contact with CyD [43]. In some other complexes the CyD appears as a coordinating ligand [44-49]. For example the Imidazole-appended β-CyD forms a ternary complex with a Cu$^{2+}$ ion and l-tryptophanate [50]. The 6-amino and imidazolyl groups of the host molecule and the carboxyl and amino groups of l-tryptophanate are coordinated to the Cu$^{2+}$ ion.

Moreover the cavity microenvironment of CyDs may alter the rate constant of reactions for the guest molecules depend on the reaction, substrate and the differences between cavity and solvent environments [51-53]. The changes in reaction rate cause to spectral time profile of the substrate and may be applicable in selective kinetic measurement of substrates and their recognition [54, 55].

### 2.4. Luminescence based molecular recognition

CyD inclusion is a means for protection of an excited state luminescent guest from the solvent environment that frequently shows a marked increase of luminescence due to increase in quantum yield and lifetime [56]. It have been mentioned even in some textbook that addition of CyD in solution is an efficient way in attaining the room temperature phosphorescence.
effect is usually much larger than that observed in absorption, and has therefore been used more efficiently and sensitively for luminescing substrates. 6-bromo-2-naphthol is a good example that exhibited room temperature in the presence of β-CyD owing to protection from \( \text{O}_2 \) quenching in a nondeoxygenated solution, although nitrogen purging increased the emission intensity 13-fold [57].

For 2-chloronaphthalene solutions containing both d-glucose and α-CyD, the room-temperature phosphorescence of 2-chloronaphthalene has been observed. The 2:1 inclusion complex is responsible for the room-temperature phosphorescence. The quantum yield of the room-temperature phosphorescence from the 2:1 inclusion complex has been determined to be 19% of alcoholic solution at 77 K. When KI is added an enhancement is observed in phosphorescence intensity due to the formation of a ternary inclusion complex with iodide. Also the intensity reduction at higher concentrations of KI seems to be due to the formation of a nonphosphorescent ternary inclusion complex containing two iodides [58]. The notion of “turn-on” fluorescent sensor is used for this molecular recognition mechanism.

For the crown ether fluoroionophore/β-CyD complex, the dimerization of the fluoroionophore inside the β-CyD is found to be selectively promoted by alkali metal ion binding, thereby resulting in metal-ion-selective pyrene dimer emission in water. This supramolecular function is successfully utilized in the design of a podand fluoroionophore/β-CyD complex for sensing toxic lead ion in water [59, 60].

Figure 11. Response mechanism of benzo-15-crown-5 fluoroionophore/γ-CyD complex for K⁺ in water.

A further interesting application of fluorescence spectroscopy is its potential enantioselectivity. Chiral discrimination has been demonstrated for CyD inclusion of camphorquinone [61]. The measurement of fluorescence anisotropy has been proposed as a method to determine the enantiomeric composition of samples [62].

As well as UV-Visible spectroscopy; competition of desired analyte with CyD-bonded or dissolved fluorophore yields a significant change in the fluorescence signal that will be useful in molecular recognition. Various “turn-off” fluorescent chemical sensors, in which fluorescence intensity was decreased by complexation with guest molecules, were reported.
A comprehensive example molecular recognition based on both decrease and increase in fluorescence intensity is the dansyl bonded CyD with diethylenetriamine spacer (CyD-dien-DNS) which have been reported by Corradini et al. In the presence of lipophilic organic molecules, CyD-dien-DNS showed sensing properties due to competitive inclusion of the guest and “in-out” movement of the dansyl group. CyD-dien-DNS was found also to be a fluorescent chemosensor for copper(II) ion, with a linear response and good selectivity, suggesting that a more flexible conformation of the linker and the presence of additional binding sites allow binding of the metal ion by the amino and sulfonamidate groups.

The CyD-dien-DNS copper(II) complex was shown to behave as a chemosensor for bifunctional molecules, such as amino acids. In fact, upon addition of alanine, tryptophan, and thyroxine, the negligible fluorescence intensity of Cu(CyD-dien-DNS) complex was “switched on”, with a response dependent on the amino acid side chain [63]. Fluorescent indolizine
modified CyD were studied in aqueous solution to evaluate their potentialities as molecular chemosensors for volatile organic compounds (VOCs) such as adamantanol, benzene, toluene, phenol and p-cresol as guest. The formation constant values measured using a spectral displacement method and also some specific algorithm treatments are reported for their quantitative analysis. [64, 65]. Some phenylseleno derivatives of CyD have been synthesized as chiral molecular sensors. These modified cyclodextrins can recognize both the size and chirality of the guest molecules despite of this fact that their stability constants with aliphatic alcohols are generally smaller than those for native β-CyD [66].

Moreover some chiral amino acid modified CyDs have been synthesized as chiral molecular sensors. N-dansyl-L-Phe-modified β-CyD showed high D-selectivity for norbornane derivatives and N-dansyl-D-Phe-modified β-CyD showed high L-selectivity for menthol [67]. Time-resolved fluorescence studies showed that the fluorescence of the dansyl group was completely quenched in the ternary complexes formed, and that the residual fluorescence was due to uncomplexed ligand. The enantioselectivity in response was found to be due to the formation of diastereomeric ternary complexes [68,69]. Fluorophore-amino acid-CyD were synthesized and characterized as fluorescent indicators of molecular recognition [70]. A novel boronic acid fluorophore 1/β-CyD complex sensor for sugar recognition in water has been designed [71].

2.5. Recognition of toxins based on spectral changes

There are also some successful applications of CyDs based spectral changes which have been used for the recognition of biologically important toxins.

Cyanotoxins are potent toxic compounds produced by cyanobacteria during algal blooms, which threaten drinking water supplies. These compounds can poison and kill animals and humans. The host–guest interactions of CyDs with problematic cyanotoxins were investigated to demonstrate the potential application of CyDs for the removal of these toxins from drinking water or applications related to their separation or purification. The complexation of these cyanotoxins with CyDs was monitored by nuclear magnetic resonance (NMR). The observed changes in chemical shifts for specific protons and competitive binding experiments demonstrate a 1:1 inclusion complex between γ-CyD and microcystins and nodularin, and the results suggest that CyD-type substrates are useful hosts for their complexation [72].

The fluorescence properties of the aflatoxins, as the most important mycotoxins, and the effect of various CyDs on their fluorescence emission were studied. The complex formation constant \( K_f \) of these compounds with β-CyD was chromatographically determined, and from the results obtained, it has been concluded that \( K_f \) cannot be used alone to explain the fluorescence increase [73].

An example of determination of biological toxins is a highly sensitive and rapid strategy for characterizing aflatoxins and the cholera toxin based on capillary electrokinetic chromatography with multiphoton-excited fluorescence. The aflatoxins are a highly mutagenic multiple-ringed heterocycles produced by aspergillus fungi and cholera toxin a-subunit is the catalytic domain of the bacterial protein toxin from Vibrio cholera. The anionic carboxymethyl-β-CyD, used to chromatographically resolve the uncharged aflatoxins, enhances emission from these
compounds without contributing substantially to the background [74]. Also the determination of aflatoxin B1 (AFB1) in wheat has been accomplished by enhanced spectrofluorimetry in the presence of β-CyD. The method is based on the enhanced fluorescence of AFB1 by β-CyD in 10% (w/w) methanol–water solution. The adopted strategy combined the use of parallel factor analysis (PARAFAC) for extraction of the pure analyte signal and the standard addition method, for a determination in the presence of matrix effect caused by wheat matrix [75].

Figure 14. Contour plots (excitation–emission) for an original wheat sample and four AFB1 standard additions; (a) the original sample, (b) plus 2.0 µg kg\(^{-1}\), (c) plus 3.8 µg kg\(^{-1}\), (d) plus 5.7 µg kg\(^{-1}\), (e) plus 7.4 µg kg\(^{-1}\). [Reprinted from Hashemi J, Asadi Kram G, Alizadeh N. / Talanta, 2008; 75 1075-1081 with permission from Elsevier Science.]
2.6. Interaction and recognition of natural compounds

Finally the spectral change and interaction of some natural compounds such as alkaloids and peptides with CyDs is discussed.

Complex formation of the glutathione and some of its derivatives with bridged β-CyD such as 2,2′-diseleno-bridged β-CyD were determined by UV-Vis. absorption and $^1$H-NMR spectroscopy [76]. Polymerization of the amyloid beta-peptide (Abeta) has been identified as a major feature of the pathogenesis of Alzheimer's disease (AD). Inhibition of the formation of these toxic polymers of Abeta has emerged as an approach for developing therapeutics for AD. NMR and circular dichroism (CD) spectra were used to investigate the interaction between CyD and Abeta. CD spectral analyses show that β-CyD inhibits the aggregation of Abeta. Analysis of the one-dimensional proton NMR spectra of the mixture of Abeta with β-CyD clearly indicates that there are chemical shift changes in the aromatic ring and the methyl groups in the peptide [77].

A series of CyDs –cinchona alkaloid inclusion complexes were prepared from β-CyD and some of its derivaties and four cinchona alkaloids, and their inclusion complexation behavior was investigated by means of fluorescence, UV/Vis and 2D NMR spectroscopy. The results showed that the cinchona alkaloids can be efficiently encapsulated in the CyD cavity in an acidic environment and sufficiently released in a neutral environment, which makes these CyD derivatives the potential carriers for cinchona alkaloids [78,79]. Using colorimetry and $^1$H-NMR and UV spectroscopy, together with solubility methods, the interaction of natural and hydroxylpropylated CyDs with xanthine, theophylline, theobromine, and caffeine in aqueous solution have been studied [80].

Combination of the spectrophotometric methods and some separation methods such as capillary electrophoresis (CE) and micellar electrokinetic chromatography (MEKC) in the presence of CyDs have been used successfully for the quantitative analysis of natural alkaloids [80,81].

3. Conclusion

CyDs are a versatile tool in the molecular recognition and sensing. Formation of inclusion complex cause to some spectral changes which have been used successfully for the study of host-guest interactions. Additionally the desired spectral changes as the results of complex formation have been used for promote analyte detection and continue to inspire creative applications. The most sensible spectral changes were reported for chemical and fluorescence indicators. These considerable changes have been used for the study and better detection of many absorbing and especially fluorescent species. Moreover many spectrochemically silent organic and some inorganic compounds cause color/fluorescence change in CyD and indicator solutions, because of their competition to form inclusion complex. These changes cause to recognition of the target competitive hosts. On this basis some “indicator modified cyclodextrin” in which indicator is linked to cyclodextrin via a spacer, was synthesized that change color/fluorescence in response to the presence of molecules, ions and many biologically
important compounds. The guest-induced changes that are roughly parallel to its binding constants were used for molecule sensing. These are valuable for qualitative and quantitative chemical analysis. Sensitivity and selectivity improved by appropriate designing of the dye moiety or spacer.

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