

Dye Encapsulation

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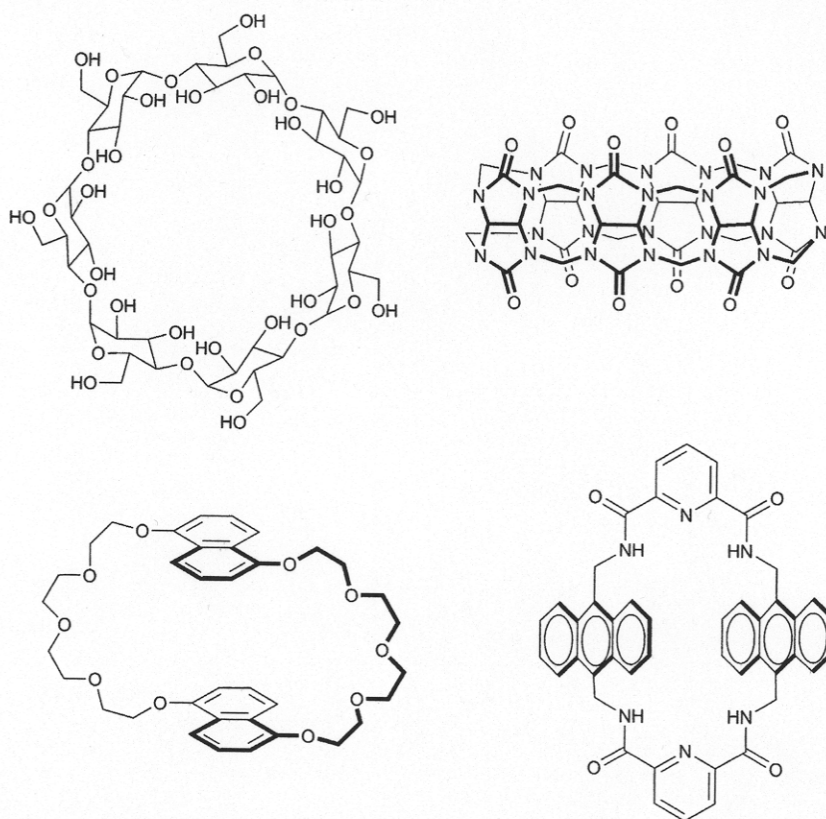
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11.1 Introduction

Organic dyes have been used throughout the ages as pigments in everyday items like paints, clothing, and printed matter. More recently, fluorescent dyes have emerged as crucial components in many high technology devices such as secure banknotes, lasing media, optical data storage, biomedical probes, and environmental sensors. A critical issue in all of these applications is dye performance, which can be judged by several parameters such as chemical stability, photochemical stability, colour intensity, fluorescence quantum yield, solubility, toxicity, etc. The systematic development of organic dyes has been pursued since the first reported synthesis of Indigo by von Baeyer in 1882,¹ an accomplishment which set in motion the German chemical industry.² In recent times, interest in fluorescent dyes has increased dramatically due to their growing impact in biotechnology and nanotechnology. It is worth noting that fluorescent dyes were a central component in the large scale sequencing methods that mapped the human genome, one of mankind's most impressive scientific achievements.³

The most obvious way to alter dye performance is to synthetically modify the covalent structure. However, despite the continued advances in synthetic organic chemistry, the process of dye synthesis is still a tedious task that consumes materials and human resources. As a way of circumventing this problem, supramolecular chemists have started



Scheme 11.1 Common container molecules: (top left) Cyclodextrin (CD), (top right) Cucurbituril (CB), (bottom left) crown ether cyclophane, (bottom right) tetralactam cyclophane

to explore non-covalent methods for altering dye properties. The focus of this chapter is on mechanical encapsulation of dyes inside: (a) protective organic container molecules such as cyclodextrins, cucurbiturils, cyclophanes, and crown ethers (Scheme 11.1), (b) biological receptors such as antibodies, aptamers and peptides, and (c) inorganic cages such as zeolites and silica nanoparticles. In some cases the dye binding process is reversible and produces a host-guest complex that is in equilibrium with the free species; in other cases the mechanical bonding is so strong that the host-guest complex can be considered a permanent molecule. Upon encapsulation, the microenvironment around the dye is changed, which often alters the absorption wavelength or the fluorescence quantum yield. Further, the dye is sterically protected, thus inhibiting intermolecular processes that often degrade dye performance like self-aggregation which reduces brightness and colour purity and photooxidation which leads to bleaching. Steric protection of long wavelength dyes is expected to be particularly helpful, because these dyes inherently have narrow HOMO-LUMO gaps and thus tend to be highly reactive.

There are various practical reasons to develop supramolecular methods of dye encapsulation. Reversible dye binding systems can be incorporated into dynamic association schemes that produce changes in colour or fluorescence emission. Thus, they can act as analytical sensing processes for environmental assays or as molecular probes for cell biology. Permanent encapsulation inside a container molecule can be achieved by several rotaxane synthesis methods that have been developed over the last twenty years.⁴ These dye rotaxanes can act as bright and highly stable fluorescent probes for diagnostics and bioimaging applications. Another way to make a bioimaging agent is to use a genetically expressible bioreceptor such as an antibody or aptamer with a recognition motif that selectively binds a dye with high affinity.⁵ These tagged bioreceptors can be visualized by fluorescence microscopy and used as imaging agents in cell biology research. Finally, permanent encapsulation of dyes inside porous inorganic matrices produces probes and advanced materials with many potential applications in biotechnology and nanotechnology.⁶

11.2 Reversible Dye Encapsulation Inside Organic Container Molecules

Organic container molecules are large macrocycles whose internal cavities can accommodate guest molecules. Historically, the most studied family of water-soluble container molecules are the cyclodextrins (CDs) which have non-polar cavities that can accommodate organic dyes of appropriate size and insulate them from an aqueous solvent. The three most common CDs are α -CD, β -CD and γ -CD with cavity diameters of 5.7, 7.8 and 9.5 Å respectively.⁷ Encapsulation of fluorescent dyes inside a CD typically improves chemical and photochemical stability and also raises the fluorescence quantum yield. However, a drawback with CD encapsulation is the relatively weak association constant ($K_a \sim 10^3$ – 10^4 M⁻¹) which can be readily disrupted. This has prompted a move in recent years to the cucurbit[n]uril (CB) family of container molecules which have much higher dye affinities in water and, like CDs, possess different diameters depending upon the number of repeating subunits in the macrocycle.⁸ For example, the association constant for the dye Rhodamine in water is 50,000 M⁻¹ with cucurbit[7]uril (CB[7]) but only 210 M⁻¹ with β -CD.⁹ The Rhodamine inclusion complex, in addition to possessing greater chemical stability and reduced dye aggregation, also exhibits properties distinct from uncomplexed Rhodamine dyes including longer fluorescence lifetimes and decreased nonspecific adsorption to surfaces.¹⁰ The inclusion complex appears quite suitable for dye based lasers, which operate most efficiently with aqueous lasing media. An aqueous solution of Rhodamine 6G complexed inside CB[7] produces better beam shape profiles when compared to free Rhodamine 6G in ethanol. CB[7] can also be used in stoichiometric ratios and thus it negligibly impacts the refractive qualities of the water, unlike detergents and emulsifiers, which also produce a dye deaggregating effect, but adversely affects performance due to bubble and foam formation.¹¹

Related studies have shown that CB[7] binds fused tricyclic dyes such as Proflavine, Pyronine Y, and Thionine with higher association constants than the analogous CD, and that dye encapsulation increases the fluorescence quantum yield.¹² In contrast to CB[7], the larger CB[8] encapsulates two dye molecules while the smaller CB[5] does not effectively encapsulate any tricyclic dye. In Figure 11.1, the perceived colour of the dye

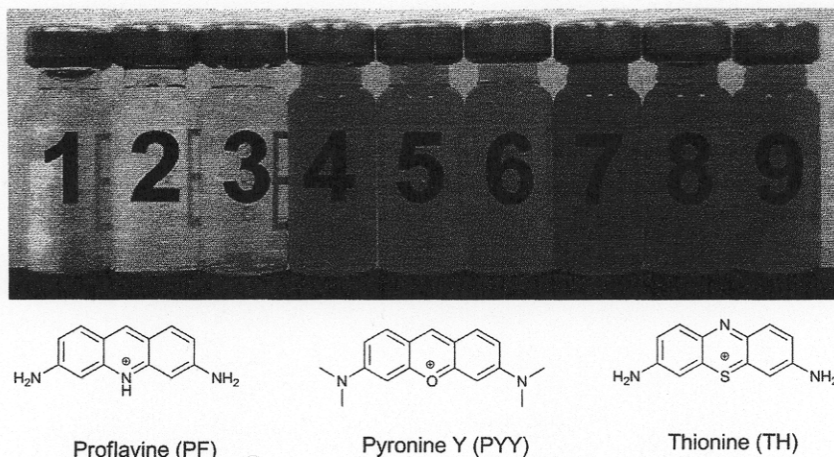


Figure 11.1 Vials containing dyes in the absence or presence of CBs. From left to right: 1) PF; 2) PF-CB[7]; 3) (PF)₂-CB[8]; 4) PYY; 5) PYY-CB[7]; 6) (PYY)₃-CB[8]; 7) TH; 8) TH-CB[7]; 9) (TH)₂-CB[8]. Reprinted with permission from [12]. Copyright 2008 Wiley-VCH Verlag GmbH & Co. KGaA

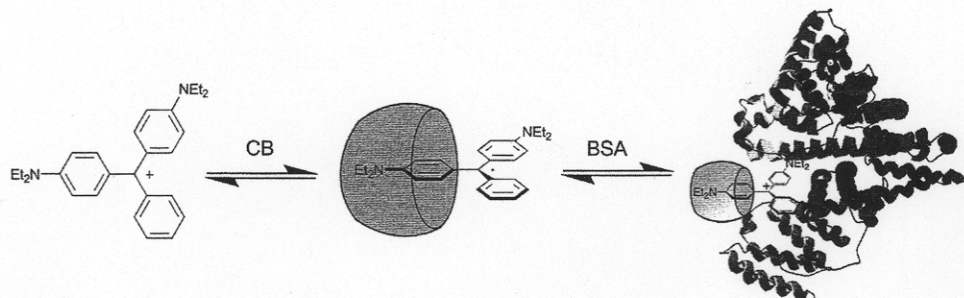


Figure 11.2 Complexation of Brilliant Green inside CB[7] causes a fluorescence increase which is further enhanced when the complex binds to the protein, bovine serum albumin. Reprinted with permission from [13]. Copyright Wiley-VCH Verlag GmbH & Co. KGaA

solution is altered by these encapsulation processes. This colour difference is attributed to changes in polarity induced by encapsulation and, when CB[8] is used, changes in dye aggregation. Separate studies have shown that CB[7] can also encapsulate Brilliant Green, a fluorescent antimicrobial trimethane dye, and form a 1:1 association complex with a binding constant of $17,000 \text{ M}^{-1}$. Intriguingly, the fluorescence quantum yield for the inclusion complex is further enhanced when it associates with bovine serum albumin (Figure 11.2).¹³

Reversible dye binding systems can be developed into colourimetric and fluorescent assays for analytical detection. As shown in Figure 11.3, two different binding schemes can be envisioned, (a) competitive dye/analyte inclusion, and (b) cooperative dye/analyte inclusion. The competitive inclusion process is the basis for the 'dye displacement assay' where the analyte displaces the dye from the container molecule. For this process to be visualized, the properties of complexed and uncomplexed dyes must be markedly dif-

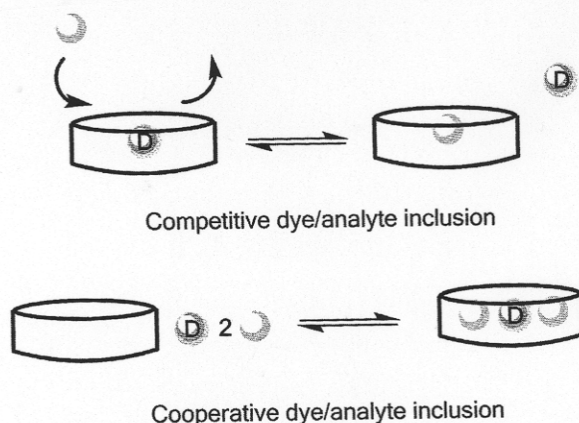


Figure 11.3 (top) Competitive dye/analyte inclusion; (bottom) cooperative dye/analyte inclusion

ferent. For example, CB[7] and the fluorescent dye Dapoxyl have been incorporated into an assay that monitors the activity of amino acid decarboxylase enzymes.¹⁴ At pH 6, Dapoxyl has a relatively high affinity for CB[7] ($K_a = 20,000 \text{ M}^{-1}$) and it is over 200 times more fluorescent when inside the CB[7] cavity. Zwitterionic amino acids have much lower binding affinities for CB[7] ($K_a < 1,000 \text{ M}^{-1}$) and cannot displace the dye, but the presence of amino acid decarboxylase leads to decarboxylated cationic products that have substantially stronger affinities for CB[7] ($\sim 30,000 \text{ M}^{-1}$). The overall scheme is a 'switch off' assay, where the product of enzyme action displaces the dye from the container molecule which decreases the fluorescence intensity. In general, 'switch on' fluorescence assays are technically easier to monitor but they are harder to devise because most dyes are more fluorescent when they are encapsulated. A rare example uses the dye 2,3-diazabicyclo[2.2.2]oct-2-ene (DBO), which is less fluorescent when it is bound to a *p*-sulfonatocalix[4]arene macrocycle.¹⁴ The action of amino acid decarboxylase leads to displacement of the DBO dye from the macrocycle and an eight-fold increase in fluorescence intensity.

The other binding scheme in Figure 11.3 is cooperative dye/analyte inclusion, where the analyte promotes association of the dye inside the container molecule. Compared to dye displacement, this is a more complicated supramolecular system because it requires the container molecule to have multi-guest recognition properties. Proof-of-concept studies have been reported using crown ether cyclophanes as container molecules that can simultaneously bind two types of guests: flat aromatic dyes and metal cations. In Figure 11.4 is a crown ether derived naphthalene macrocycle whose weak affinity for a pyromellitic diimide is increased by an order of magnitude by the presence of lithium salts.¹⁵ The crown ether oxygens coordinate to the lithium cations which in turn promotes simultaneous dye inclusion. The result is a stunning colour change from a pale yellow to bright red due to formation of a charge transfer complex. In principle, this multi-component assembly can be employed as a colorimetric sensor for lithium cations.

A related cooperative binding system is shown in Figure 11.5. A crown ether derived macrocycle only binds a squaraine dye when sodium cations are present in the solution.¹⁶ The sodium cations bridge the dye and the crown ether oxygens inside the macrocyclic

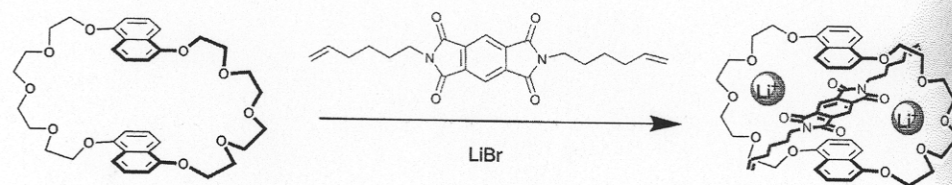


Figure 11.4 Crown ether cyclophane binds pyromellitic diimide dye only in the presence of lithium cations

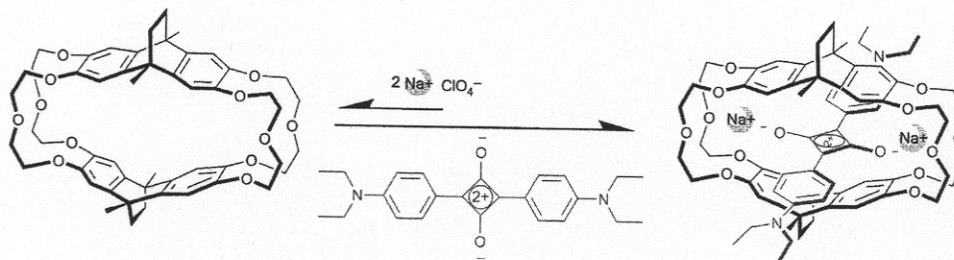


Figure 11.5 Crown ether cyclophane binds squaraine dye only in the presence of sodium cations

cavity, an inclusion process that enhances the dye's fluorescence. Addition of potassium cations leads to displacement of the sodium and consequent dye ejection from the cavity because the larger potassium ions bind to the cyclophane exterior and thus dye inclusion is no longer favoured. This multicomponent assembly system exhibits rudimentary characteristics of a combination NOT/AND logic device where the presence of sodium alone causes fluorescence signal increase, but a mixture of sodium and excess potassium or potassium alone produces no change in fluorescence.

11.3 Reversible Dye Encapsulation by Biological Receptors

There is growing interest in biological receptors for fluorescent dyes, in particular, genetically expressed receptors such as proteins or oligonucleotides.¹⁷ A major goal of this research is to discover high affinity dye recognition motifs that can be utilized for bio-imaging applications. The recognition motif can be encoded into the sequence of a larger protein or oligonucleotide of interest where the dye can be used to non-covalently label the genetically expressed target in a living cell or animal. The initial challenge is to find the appropriate dye recognition motifs using standard biotechnology screening methods such as phage display,¹⁸ yeast display,¹⁹ or SELEX (systematic evolution of ligands by exponential enrichment).²⁰ A recent example employed phage display technology to identify a 7-mer peptide sequence (IQSPHFF) with subnanomolar affinity for a fluorescent near-IR benzindolium dye (Dye A in Figure 11.6).²¹ Molecular modelling indicates that the peptide sequence forms a tweezer structure with two aromatic arms surrounding the flat dye. Incorporating the IQSPHFF sequence into larger protein structures allows

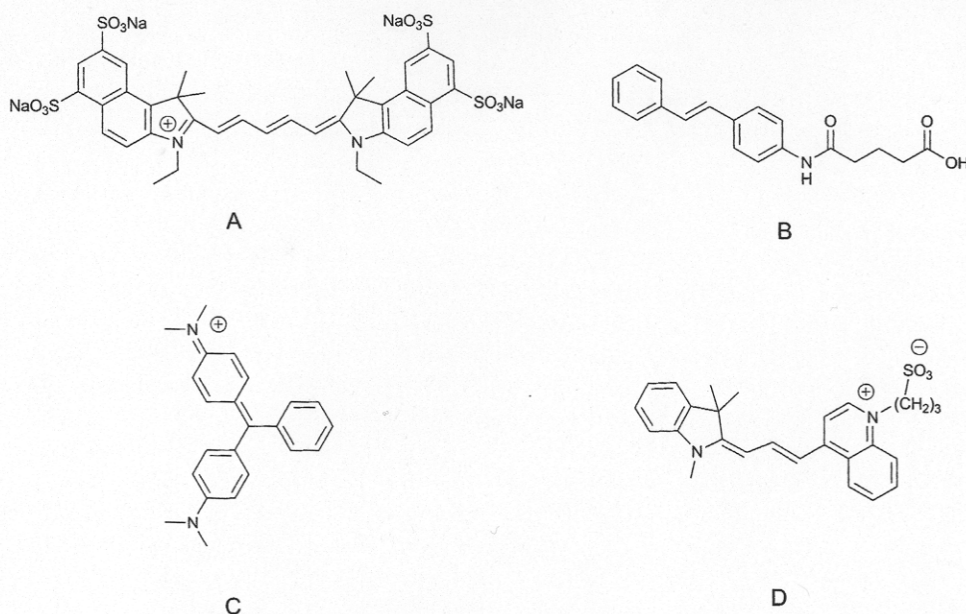


Figure 11.6 Dyes A, B, C and D

selective dye labelling in a range of assays including ELISA, flow cytometry, high throughput screens, microscopy and *in vivo* imaging. However, a drawback with this protein labelling system is the small change in the dye emission wavelength and intensity that occurs upon binding which makes it difficult to distinguish between bound and unbound dye. This means there may be an undesirably high background signal which would lower the contrast. This problem can be minimized by using a dye whose fluorescence in free solution is weak but increases substantially upon association with the bioreceptor. Several antibodies have been identified that exhibit this fluorescence activating phenomenon. One example binds a stilbene derivative (dye B in Figure 11.6) producing a blue-emitting exciplex band due to interaction of the excited state stilbene with a ground-state tryptophan residue buried in the antibody.²² Another research programme has used yeast display technology to identify single chain antibodies as fluorescence activating proteins.²³ The antibodies selectively bind target dyes such as dye C and increase the emission intensity by factors of several thousand. There has also been progress finding RNA and DNA aptamers that bind dyes and raise quantum yields.²⁴ For example, a double-stranded RNA aptamer binds the cyanine dye D with $K_d = 10^7 \text{ M}^{-1}$ and enhances the fluorescence quantum yield by 60-fold.²⁵

11.4 Permanent Dye Encapsulation Inside Rotaxanes

Permanent encapsulation eliminates any ambiguity due to partial dissociation of the dye from the host. The most common strategy for trapping a dye inside a container molecule

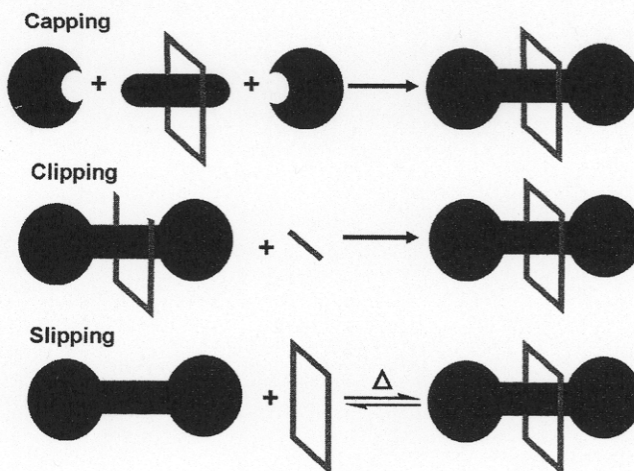


Figure 11.7 Three strategies for rotaxane synthesis: capping, clipping and slipping

is to form an interlocked structure known as a rotaxane. Rotaxane synthesis has improved greatly over the last few decades with the discovery and refinement of a number of templated reactions. The three most common methods for rotaxane formation are clipping, capping, and slipping (Figure 11.7), and each has its strengths and weaknesses. To date, much of the research effort on dye rotaxanes has focused on developing synthetic methods that are compatible with the reactive functional groups in organic dyes. Yields for the rotaxane formation step are often low (5–30%) and eventually they will have to be raised if dye rotaxanes are to be implemented in commercial applications. A promising approach that warrants further attention is to use solid-state synthesis methods (Figure 11.7).²⁶

The pioneering dye rotaxane synthesis, reported by Anderson and coworkers in 1996, utilized Glaser coupling under aqueous conditions in the presence of a water soluble cyclophane macrocycle to produce a mixture of [2] and [3]rotaxanes with a conjugated phenylacetylene fluorophore as the axle component (Figure 11.8).²⁷ The purpose of the encapsulation was to insulate the conjugated π -system from quenching processes and both rotaxanes were found to be six fold more fluorescent.

Many of the subsequent preparations of dye rotaxanes have employed CD as the protective macrocycle. In each case, a threaded complex is assembled in aqueous solution and a covalent capping reaction produces the permanently interlocked rotaxane. In Figures 11.9 and 11.10 are two capping reactions that encapsulate dyes inside CD to produce acene²⁸ and cyanine rotaxanes.²⁹ Similar methods have also been used to make phenylacetylene,³⁰ and oligo(thiophene) rotaxanes.³¹ With the larger γ -CD it is possible to simultaneously encapsulate two dyes and make homo- and hetero-[3]rotaxanes that can achieve extremely efficient energy transfer between the chromophores.³² While water is a useful solvent for driving hydrophobic dyes into the CD cavity to make the precursor threaded complexes, it does limit the scope of the capping reactions in that they must be water compatible.

The acene [2]rotaxane in Figure 11.9 was prepared by mixing an anthracene bisboronic acid with β -CD in water and conducting a Suzuki capping reaction to produce the permanent rotaxane. Unlike the free acene axle, the rotaxane does not exhibit an excimer

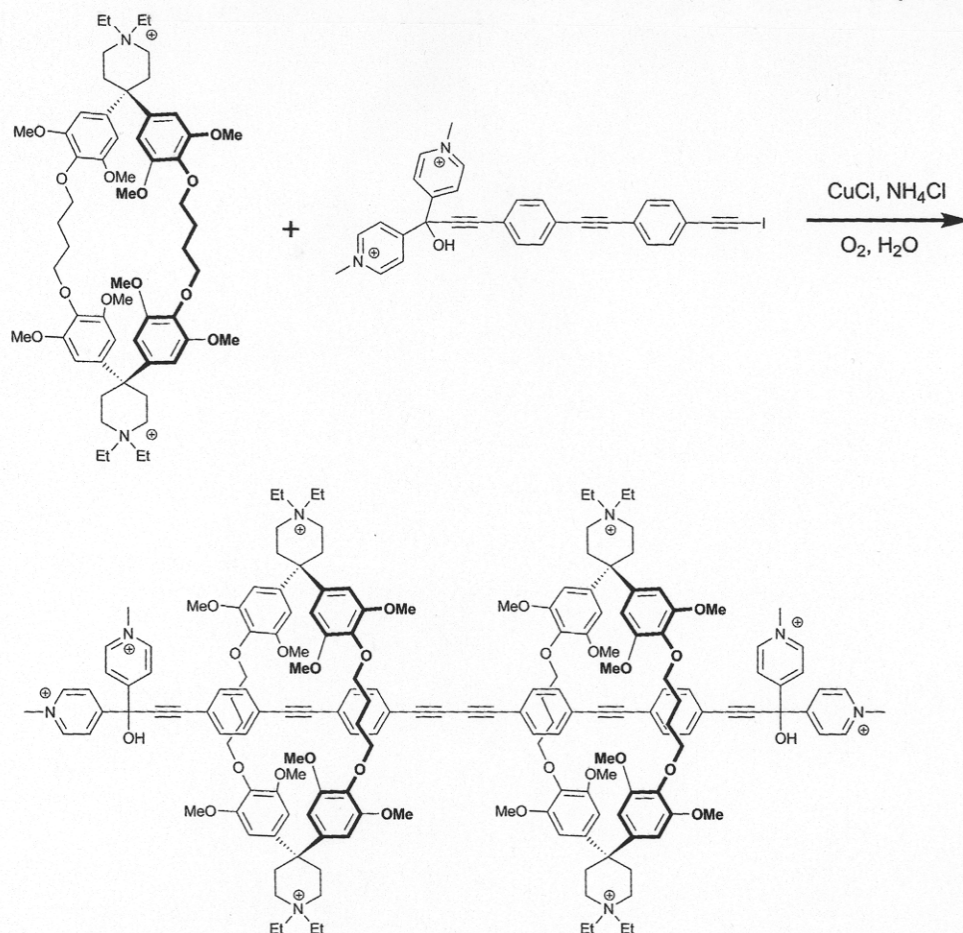


Figure 11.8 Dimerization reaction to produce a [3]rotaxane with a phenylacetylene axle

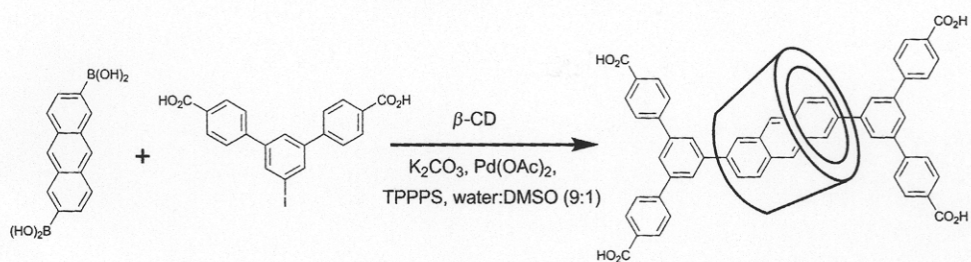


Figure 11.9 Synthesis of anthracene dye encapsulated by β -CD

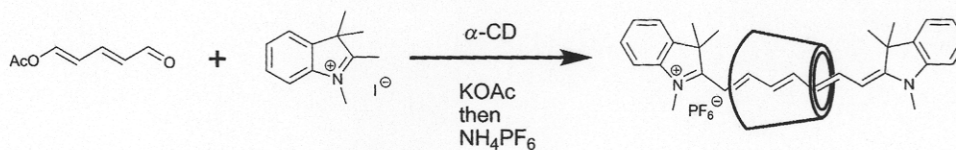


Figure 11.10 Synthesis of cyanine dye encapsulated by α -CD

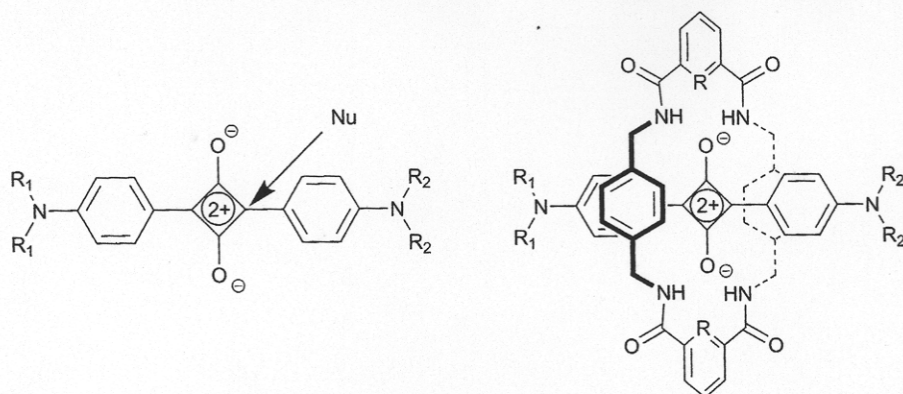


Figure 11.11 Squaraine dye is susceptible to nucleophilic attack (left), whereas the squaraine rotaxane is essentially inert (right)

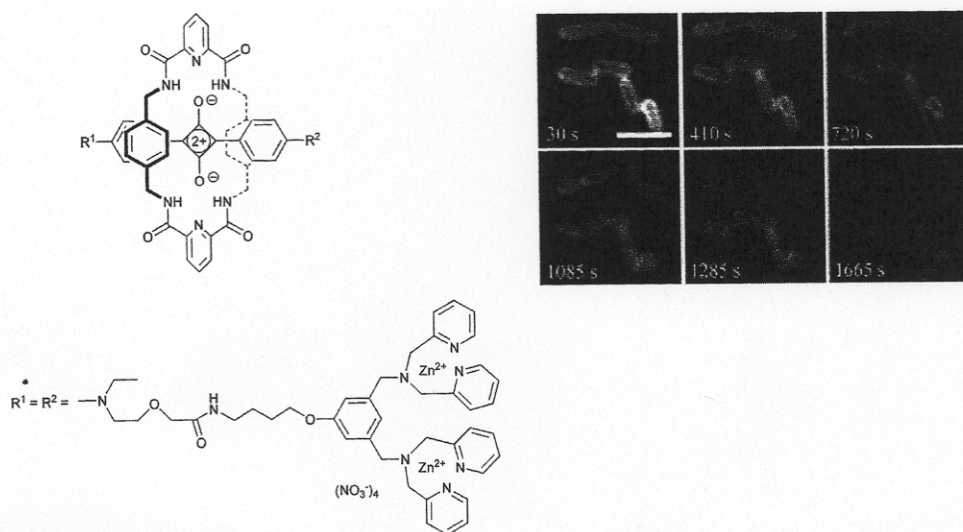


Figure 11.12 Montage from fluorescent movie showing division of bacteria cells labeled with squaraine rotaxane. Reprinted with permission from [39]. Copyright Wiley-VCH Verlag GmbH & Co. KGaA

A series of second-generation squaraine rotaxanes can be produced using the anthrylene tetralactam macrocycle shown in Scheme 11.1. The high solubility of this macrocycle in organic solvents allows squaraine rotaxanes to be produced in essentially quantitative yield by either a thermally promoted slipping process that forces the dye through the macrocyclic cavity,⁴⁰ or capping chemistry that covalently adds stopper groups to a self-assembled pseudo-rotaxane complex.⁴¹ The tetralactam macrocycle wraps tightly around the squaraine dye and there is about a 40 nm red-shift in the emission wavelength, a phenomenon that has been observed with a related perylene diimide rotaxane system.⁴²

Thus, the structure of the surrounding macrocycle is a molecular design parameter that can be used to non-covalently fine-tune the photophysical properties of the encapsulated dye.

11.5 Permanent Encapsulation Inside Inorganic Matrices

A conceptually different way to permanently encapsulate dyes is to trap them inside inorganic cages.⁴³ The most straightforward approach is to employ preformed molecular sieves or zeolites. If the dye is smaller than the diameter of the pores then solution and gas phase diffusion techniques can be used to load the dye molecules inside the zeolite (Figure 11.13). Shown in Figure 11.14 is a more sophisticated approach, which assembles the dye inside the zeolite (i.e. 'ship in a bottle synthesis') using a precursor molecule that is grafted to the cavity walls.⁴⁴ These dye doped inorganic materials can act as solar energy capture devices and as optical sensors. An example of the latter is a fluorescent humidity sensor based on Nile Red encapsulated inside NaY zeolite supercages.⁴⁵ The fluorescence of Nile Red is sensitive to solvent polarity and incorporation within the zeolitic pores further enhances this effect, partly because the zeolite cavities are hydrophilic.

Clay materials, like zeolites, have also been explored as naturally occurring microporous hosts for dyes.⁴⁶ Trapping the dyes within these three dimensional scaffolds provides protection from external quenchers but inhibits aggregation behaviour by immobilizing and segregating them within the framework of the material. Interestingly, dye encapsulation within micro and mesoporous materials was performed by the ancient Mayas several thousand years ago. Indigo, a naturally derived blue dye extracted from many plants of the genus *Indigofera*, has modest stability but encapsulation inside palygorskite clay produces a dye of exceptional stability, one that is inert to high and low pH solutions, photobleaching and oxidation. The packing arrangement of the dye within the clay is still a matter of investigation but computational and spectroscopic evidence suggests the zeolitic water in the clay is released upon inclusion of indigo and H-bond contacts

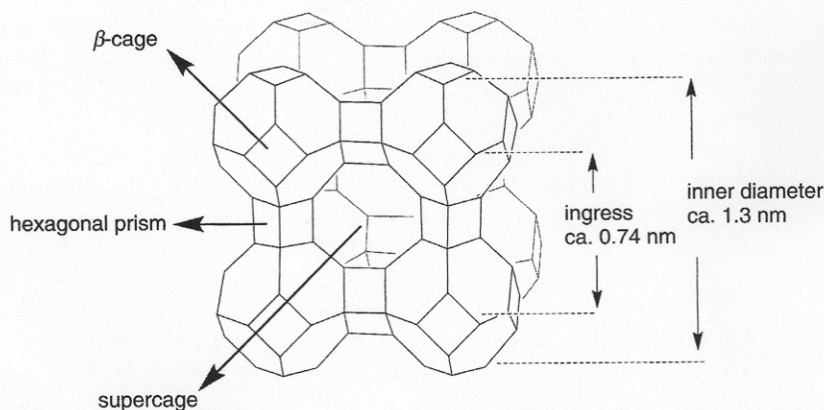


Figure 11.13 Conceptualization of a microporous zeolite. Adapted from Reference 46

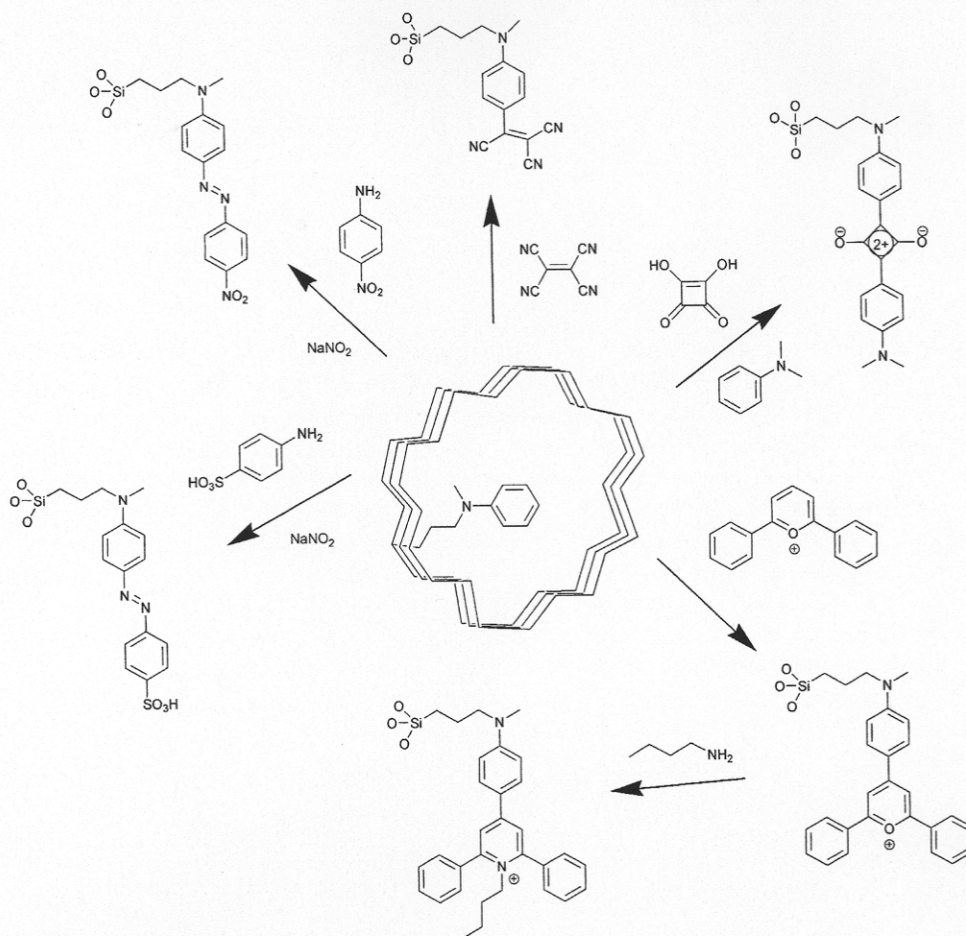


Figure 11.14 Dye synthesis inside silica cage. Adapted from Reference 44

between the dye walls of the clay and structural water molecules (those molecules that make up the scaffold of the clay) form stabilizing interactions.⁴⁷

The desire to produce non-toxic fluorescent nanoparticles for bioimaging and diagnostic applications has led to new methods of doping dyes within the lattice structures of silica nanoparticles.⁴⁸ The trapped dyes exhibit enhanced stability, increased fluorescence, and decreased self-aggregation. A range of related microemulsion methods have been reported to produce dye-doped and core-shell silica nanoparticles.⁴⁹ In some cases, the dyes are covalently conjugated to the silicate precursor which leads to greater dye dispersal within the nanoparticle.⁵⁰ An alternative method employs a cationic surfactant to template the formation of mesoporous silica containing the molecularly dispersed dyes. Fluorescent silica nanoparticles are potentially useful as sensors for lab-on-chip diagnostics and as probes for cell and animal imaging. Nanoparticle probes that contain photosensitizing dyes are potential candidates for photodynamic therapy.⁵¹ Most photosensitizing

dyes are hydrophobic, which leads to formulation problems and poor *in vivo* distribution. Covalently attaching photosensitizers to the nanoparticles enables convenient delivery to tumor cells without the problem of dye diffusion away from the target site. Molecular oxygen can penetrate the pores of the nanoparticles, thus irradiation of the target site after nanoparticle delivery leads to singlet oxygen release within the tumor. Additional cell targeting properties can be acquired by decorating the nanoparticle surface with affinity ligands or cell penetrating peptides.⁵²

11.6 Conclusion

Most of the organic dyes that are currently employed in colourimetric and biosensing assays have been around for many decades and although they do not always have optimal performance properties they remain useful because they have low toxicity and relatively low cost. The emergence of new technologies in frontier industries like biomedicine, materials science, and nanotechnology creates a growing demand for dyes that have improved performance. One of the major attractions of molecular encapsulation is that it uses established, familiar dyes as central chromophores; however, a potential drawback with some applications is that the encapsulation matrix will add considerable size and mass to the final product. In most cases, the crucial factor that will determine the eventual scientific and societal impact of encapsulated dye products is the economic cost of fabrication, and there is a continuing need to develop high-yielding templated synthesis methods. The remarkable synthetic success that has been achieved in recent years is reason to expect further major progress. Intellectually, dye encapsulation is likely to expand as an interdisciplinary field, connecting fundamental scientists and engineers who aim to invent next-generation nanoscale devices and molecular machines.

Acknowledgements

We are grateful for financial support from the NIH and the NSF (USA).

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