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Optical Probes for the Detection of Protons, Alkali and Alkaline Earth metal Cations

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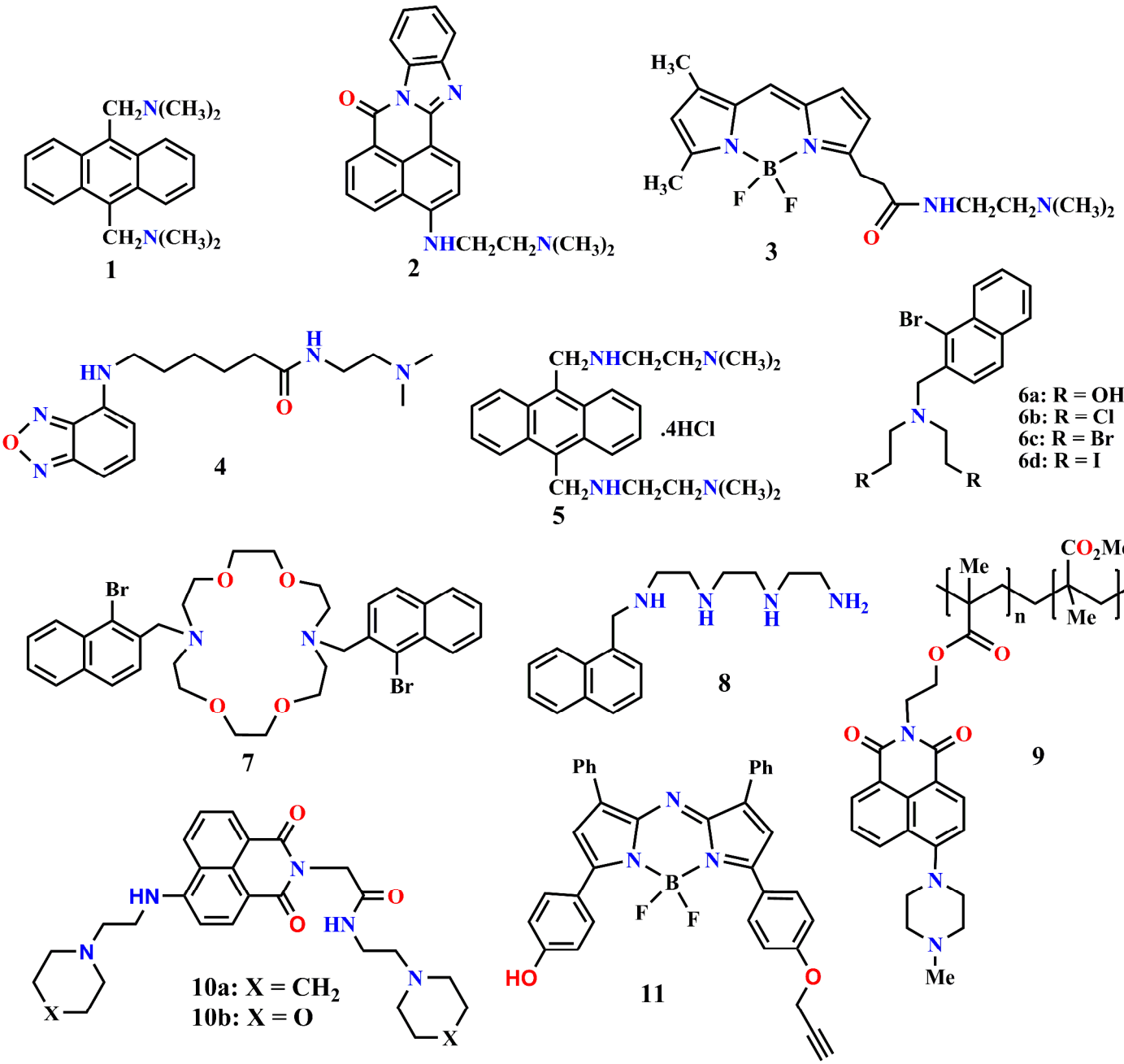
Abstract: Luminescent sensors and switches continue to play a key role in shaping our understanding of key biochemical processes, assist in the diagnosis of disease and contribute to the design of new drugs and therapies. Similarly, their contribution to the environment cannot be understated as they offer a portable means to undertake field testing for hazardous chemicals and pollutants such as heavy metals. From a physiological perspective, the Group I and II metal ions are among the most important in periodic table with blood plasma levels of H^+, Na^+ and Ca^{2+} being indicators of several possible disease states. In this review, we examine the progress that has been made in the development of luminescent probes for Group I and Group II ions as well as protons. The potential applications of these probes and the mechanism involved in controlling their luminescent response upon analyte binding will also be discussed.

Keywords: Probe, Sensor, Fluorescence, Phosphoresence, PET, FRET, Excimer, Proton, Alkali, Alkaline Earth metal cation.

1. Introduction: Given the physiological relevance of protons and metal cations such as sodium, potassium and calcium, it is perhaps not surprising that they remain among the most popular targets for the development of new optical probes. The past decade has witnessed significant activity in the development of sensors, switches and molecular machines for the detection of such analytes and has been the subject of many different reviews.[1] These probes need to demonstrate high selectivity for the target analyte over other possible interfering analytes, have appropriate binding constants to enable operation in the desired range, and possess good biocompatibity if needed for use in biological environments. This review will provide an overview of the progress made in developing probes for protons, alkali and alkaline earth metal ions that operate primarily through a change in their optical signature between the analyte bound and unbound states. The mechanisms used to enable such as change in optical signature will also be discussed.

2. Proton (H^+) Recognition: A number of key biochemical processes are dependent upon proton concentration and deviations from the normal range can lead to health related problems such as cancer and certain neurological disorders.[2] Deviations in proton concentration can also have a detrimental effect on the environment *e.g.* acidification of rivers, seawater and lakes by acid rain or contamination.[3] In some respects, protons represent the easiest target for sensor development due to their small size and attraction to basic receptors such as amines. Indeed, tertiary amines form the core unit of many photoinduced electron transfer (PET) based receptors as the thermodynamic driving force (ΔG_{PET}) for electron transfer can easily be overturned through chelation of the amine lone pair to protons or metal ions.[4] Not surprisingly, therefore, proton interference can also be a major challenge in the design of metal ion and small molecule optical probes and careful attention must be paid to the pKa of the basic units used in these receptors.

Endocytosis is a cellular process whereby large polar molecules are engulfed by the cell membrane and transported within the cell.[5] The molecules begin their journey in early endosomes that progress to late endosomes and finally lysosomes. The process is characterized by a lowering of pH so that in late endosomes and early lysosomes a value as low as pH = 5 is typical.[6] Fluorescent probes have proven useful tools in following the endocytosis pathway and commercially available probes are available under the trade names Lysosensor™ Blue (**1**) and Green (**2**)™ and Lysotracker™ Green (**3**), Yellow (**4**) and Blue (**5**).[7] These probes typically contain a fluorophore connected to a tertiary amine receptor via a spacer group and operate according to the PET mechanism.[8] The pKa of the tertiary amine is usually between 4-5 so that at normal cellular pH fluorescence is low due to PET from the amine nitrogen lone pair to the fluorophore that quenches fluorescence. In acidic organelles, the amine becomes protonated increasing its oxidation potential making PET energetically unfavorable and switching fluorescence “On”.



In a continuation of this approach Wang *et al.* prepared derivatives **6**, **7** based on the 2-bromomethyl naphthalene (BMN) phosphor.[9] The bromine atom present on BMN promotes intersystem crossing through a heavy atom effect leading to triplet state emission. Following a phosphor-spacer-receptor approach, the authors attached various substituents (-OH, -Cl, -Br and –I) to the tertiary amine receptor, presumably to modify the pKa through inductive effects. The authors added the derivatives of **6** to an aqueous solution containing β-cyclodextrin (β CD) and found that the phorophorescence intensity increased with increased β CD concentration but the effective formation of 1:1 binary complexes were also influenced by the size of the substituent present on **6**. The intensity of phosphorescence emission was also dependent on solution pH with low pH switching

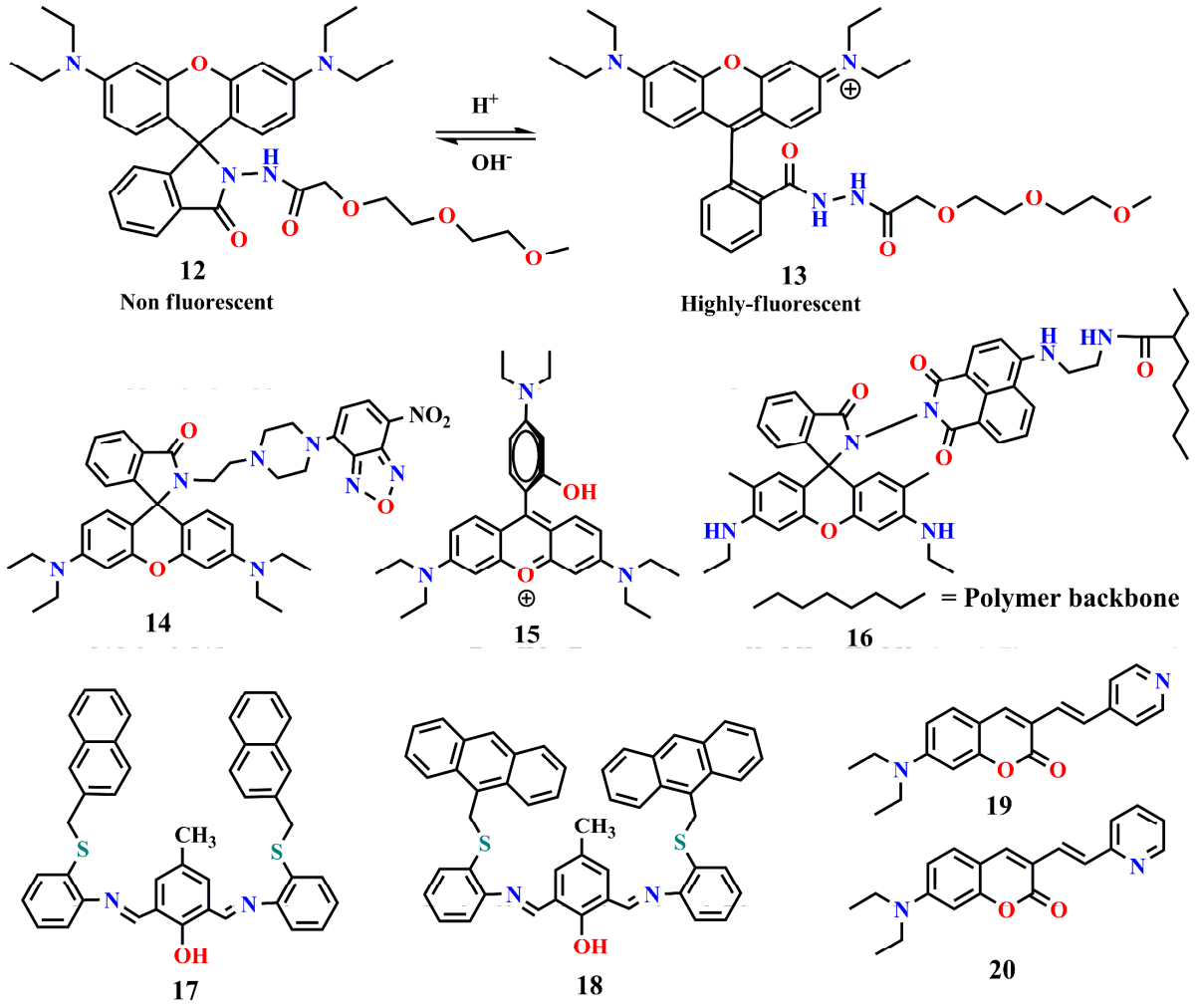
fluorescence "On" although there was no significant change in pKa between halogen substituted **6b-6d**. Surprisingly, azo-crown containing **7** did not show any interference when tested against a range of metal ions.

While the interactions of H^+ with naphthylmethylpolyamines were studied during the formative phase of PET sensors, Pina *et al*. cleverly analyse the PET rates from **8** and its relatives as a function of the electron-transfer distance.[10] The former was derived from fluorescence lifetime measurements, while the latter was obtained from NMR data. This work contributes to the understanding of the distance dependence nature of PET,[11] and so molecules like **8** enjoy provide vital information to the field.[12] Tian *et al*.'s copolymer **9**[13] includes the 'fluorophore-spacer-receptor' common with PET systems where H^+ binding to the tertiary aliphatic amine inhibits PET and switches on the emission of the naphthalimide unit.[14-16] This phenomenon is cleverly combined with photogenerated acids, from precursors such as triphenylsulfonium salts, to achieve 'light writing' of fluorescent images. Gunnlaugsson's **10** also operates according to the PET mechanism and possesses either a piperidine (**10a**) or morpholine (**10b**) receptor to modify the pKa value.[17] The probes where then incorporated within a mixed poly(methylmethacrylate) (MMA) poly(hydroxyethylmethacrylate) (HEMA) hydrogel. The resulting hydrogels were shown to be responsive to pH with a significant enhancement in fluorescence when they were bathed at pH 2.5 compared to 11.5. Given the non-covalent attachment between the probe and hydrogel matrix it would have interesting to determine how much of the probe that would leach from the hydrogel over time. Nonetheless, incorporating optical probes with polymer matrices have obvious potential applications as "smart materials".

A relatively recent fluorophore to emerge in the pH sensing arena is the BF_2-chelated tetraaryldipyromethene unit developed by O'Shea and co-workers.[18] This fluorophore boasts impressive absorption (688 nm) and emission maxima (716 nm) with a quantum yield of 37%, and in the case of **11**, the authors were able to control emission through a phenol / phenolate inter-conversion. **11** was operable in the physiological pH range with a pKa of 6.9. and also contained an alkyne moiety enabling it to be functionalised with a wide range of azide containing substrates in a facile manner with no loss in spectroscopic properties. The ability of **11** to switch its fluorescence in MDA-MB-231 cells where the pH was adjusted from 6.6 to 8.0 was also proven.

An alternative strategy that has been adopted for pH sensing that does not involve PET is the H^+ mediated conversion of the ring closed spirolactam (non-fluorescent) to its ring opened amide (fluorescent) analogue. The perceived benefits of this approach are (i) the residual fluorescence sometimes observed by inefficient PET quenching resulting in background interference is overcome and (ii) the basic nature of amine containing probes may increase the pH of acidic organelles over time. Therefore, this approach has received significant interest in the pH sensing arena. Peng *et al.* developed rhodamine-lactam **12**, comprising a spirolactam ring, attached to a methyl carbitol chain whose function was to serve as a lysosomal marker.[19] Indeed, absence of the carbitol

chain reduced the ability of **12** to identify acidic organelles. It was found that **12** was completely stable at pH 7.4 and showed no fluorescence due to the closed spirolactam ring, but upon contact with H^+ ions the fluorescence increased remarkably (> 50 fold at pH 4.5) to form **13**. Due to the nature of the mechanism involved, it is no surprise that **12** displayed good selectivity for protons over other competing metal ions.



Following a similar approach, Xu *et al.* prepared rhodamine B based **14** in a two-step reaction using 1-(2-aminoethyl)piperazine, rhodamine B and 4-chloro-7-nitro-2,1,3-benzoxadiazole.[20] They tested the prepared probe with various cations but no changes in spectral profile was observed. However, addition of H^+ ions produced a considerable change in spectral profile of **14**, with the appearance of a new blue-shifted peak at 490 nm. The change in spectral profile was again attributed to opening of spirolactam ring in the presence of protons.

Suna *et al.* prepared rosamine based probe **15**, a rhodamine analogue based pH probe by a one-step synthesis using *m*-diethylaminophenol, trimethyl orthoformate and pyridinium p-toluenesulfonate.[21] The probe was non-fluorescent at pH = 8, but red fluorescence with a 400 fold enhancement in intensity was observed

when the pH was lowered to 3. The probe was highly selective for protons over a range of biologically relevant metal ions and used to determine the pH of acidic organelles in HeLa cells.

Bojinov *et al.* reported the novel FRET (Förster Resonance Energy Transfer) based pH probe **16** using a 1,8-naphthalimide–rhodamine bichromophore system.[22] The bichromophore system was covalently attached to amphiphilic copolymer, based on poly(methyl methacrylate)-*b*-poly(methacrylic acid) (PMMA-*b*-PMAA) that self-assembled to form micelles in aqueous media. Ring-opening of the spirolactam ring upon protonation results in the evolution of a strong rhodamine absorbance at 532 nm that overlaps the napthalimide emission band encouraging FRET between the two units. The outcome is strong rhodamine emission upon excitation of the natpthalimide component.

Das *et al.* synthesized **17** and **18** for the detection of H^+ ion concentration over different pH ranges.[23] Like **16**, these FRET based probes were based on the principle that the acceptor absorbance spectrum changes with H^+ concentration. The use of two different acceptors in napthalene (**17**) and anthracene (**18**), both with distinctly different emission spectra (centered at 375 nm and 450 nm respectively), in combination with the same donor (p-cresol) modifies the natural donor / acceptor spectral overlap in **17** and **18**. Additionally, protonation / de-protonation of the p-cresol phenolic unit alters the position of the acceptor absorbance from 350 nm in acid to 450 nm in base. Therefore, acidic conditions favor FRET in **17** while basic conditions do so in **18**. Together, these probes were capable of measuring pH over a very broad range.

Coumarin based ratiometric H^+ sensors **19** and **20** were prepared by Long *et al.* utilizing pyridine as the receptor for H^+.[24] The absorption and emission maximum of both probes were red shifted upon increasing H^+ concentration with enhanced intensity. Moreover, the observed emission ratio (I_{529}:I_{616}) was linear as a function of pH in the 4.0 to 6.5 range. NMR experiments and DFT studies confirmed binding of H^+ bind to nitrogen atom of pyridine, altering the internal charge transfer (ICT) process leading to the observed red-shift. These sensors were also successfully utilized for sensing H^+ concentration in newborn calf serum and urine as well as effectively monitoring H^+ concentration in living cells.

Overall, acid–base interactions remain the simplest, most convenient and most defined testing ground for sensor designers.

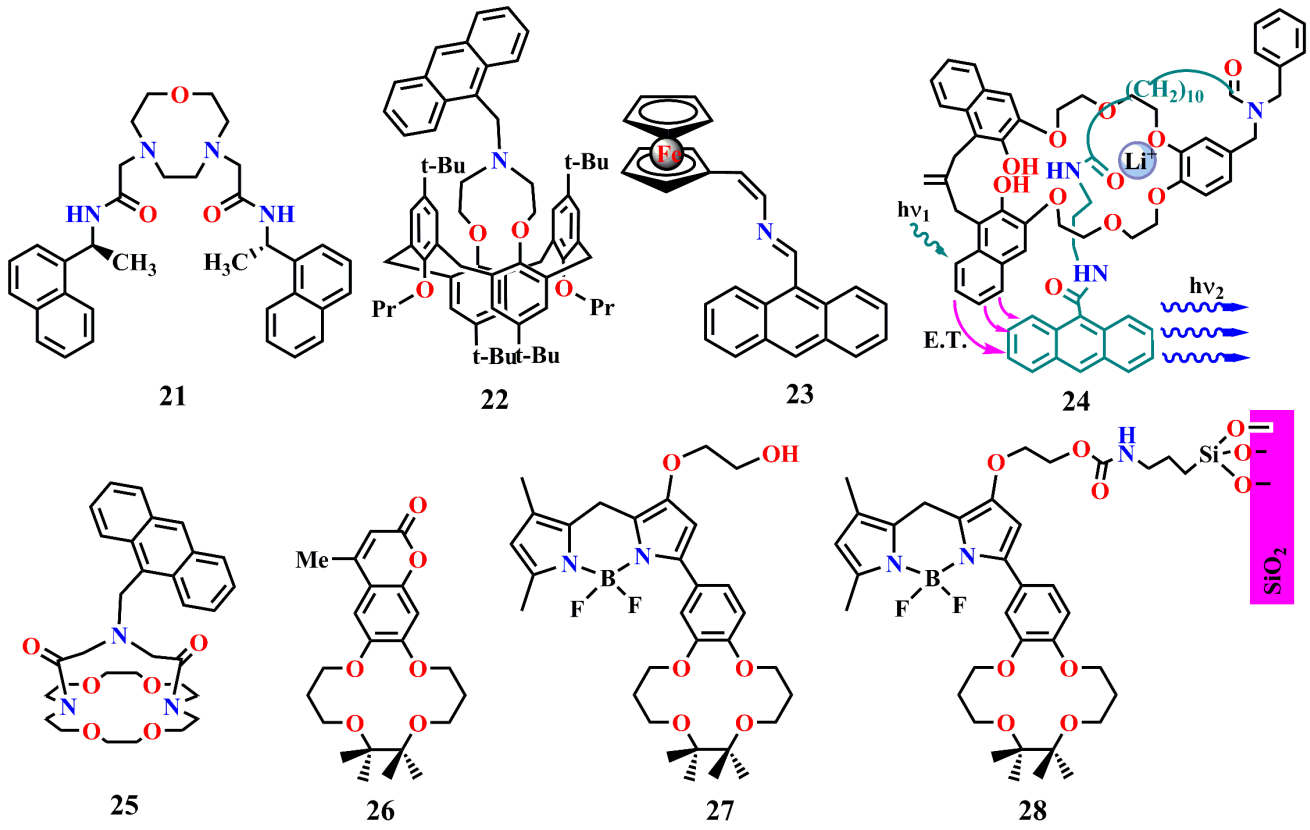
3. Alkali Metal Ions

3.1. Sensors for Li^+: Lithium salts are widely used in the treatment of certain psychiatric disorders such as manic-depressive psychosis. After administration of drugs containing Li^+, its plasma concentration should be

tightly controlled in the 0.6-1.2 mM range, as excessive amounts may cause serious detrimental effects, possibly leading to death.[25,26] In recent years, numerous fluorescent chemosensors have been developed for the selective detection of Li^+ ions. However, as other co-existing alkali (i.e. Na^+ and K^+) and alkaline earth metal ions (i.e. Ca^{2+}) are present at even higher concentrations in blood compared to Li^+, the design of suitable methods for monitoring of Li^+ is very challenging.

Crown ethers have proven popular receptors for alkaline metal ions due to the various possible cavity sizes available and the effective chelation ability of the oxygen lone pairs for these "hard" metal ions. Parker and co-workers demonstrated the effectiveness of these ligands for the selective detection of Li^+ in blood serum using potentiometric detection.[27] This work was extended by Gunnlaugsson *et al*. who utilized diaza-9-crown-3 as a small binding pocket receptor for Li^+ which was connected with two naphthalene fluorophores in a PET format **21**.[28] In CH_3CN, the fluorescence modulation (9 fold) of **21** at 337 nm was observed selectively in the presence of Li^+. Subsequently, the basis of a size-fit effect approach was adopted for the selective detection of Li^+ by using the calix[4]arene based compound **22** with a smaller binding pocket 'azacrown-3' connected to an anthryl fluorophore.[29] In CH_2Cl_2/THF (75:25) medium, **22** detects Li^+ selectively with a fluorescent enhancement of greater than 106-fold and excellent selectivity over Na^+ and K^+ (log $K_{Li,Na}$ = -3.8 and log $K_{Li,K}$ = -2.3, where $\log K_{i,j} = \log([i]/[j])$ with j being the concentration of interfering ion that provides maximum fluorescence response and i the concentration of the analyte ion that produces the same fluorescence response produced by the interfering ion). However, the inability of **21** and **22** to operate effectively in aqueous medium has obvious restrictions.

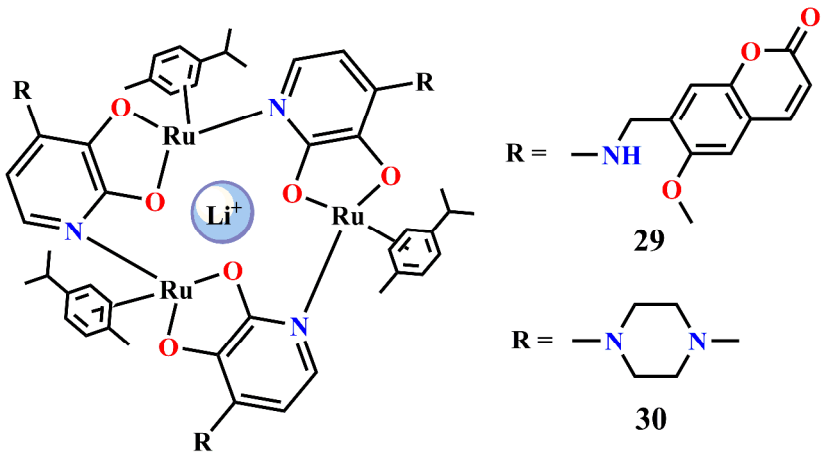
An alternative approach to enable effective sensing of Li^+ in aqueous medium was reported by Caballero et al., who combined anthracene and ferrocene units via a 2-aza-1,3-butadiene bridge **23**[30] for the highly selective sensing of Li^+ in CH_3CN/H_2O (70/30). At pH = 5, when the imine-N is protonated, the fluorescence of **23** was selectively enhanced in the presence of Li^+ over the other tested metal ions (Na^+, K^+, Ca^{2+}, Cu^{2+}, and Zn^{2+}) with an association constant (K_a) of 11.757± 0.019 M^{-1}. DFT calculations suggested the Li^+ ion formed a 1:1 host-guest complex with ferrocene unit of **23** that was stabilized by hydrogen bonding with two water molecules, while the nitrogen atom in the 1,3-butadiene bridge was protonated . Indeed, the requirement of both a Li^+ ion and a proton to be bound before strong anthracene fluorescence was observed suggest two quenching channels being present in **23**. While effective at binding Li^+ selectively in semi-aqueous solution, the requirement of a pH two units lower than physiological pH is not ideal.



FRET based system **24** was designed for the fluorescent sensing of Li^+ in CH_2Cl_2/CH_3CN (90:10) medium.[31] The fluorescence spectrum of free **24** displayed an emission between 400-500 nm ($_{exc}$ = 285 nm). Upon addition of alkali metal ions (Li^+, Na^+ and K^+), the fluorescence intensity of **24** was selectively and remarkably enhanced in the presence of Li^+ by forming a 1:1 host-guest complex with a binding constant (K_a) of 8.4 ± 0.3 x 10^3. NMR studies suggested the Li^+ ion was selectively encapsulated inside the three-dimensional rotaxane cavity of **24**, causing a conformational change that brought two fluorophores (naphthalene and anthracene) closer together with the orientation for effective energy transfer.

By modifying the rigid binding pocket present in **22** to a less rigid structure in **25**,[32] a 190 fold fluorescent enhancement was obtained upon addition of Li^+ ion to a CH_2Cl_2/THF (75:25) solution containing **25**. However, the relative selectivity of **25** for Li^+ over Na^+ (log $K_{Li,Na}$ ~ -3.36) and K^+ (log $K_{Li,K}$ ~ -1.77) was slightly lower than for **22**. However **26**, containing a'14-crown-4' receptor and 4-methylcoumarin fluorophore was effective at selectively sensing Li^+ in an almost 100% aqueous based solvent (water/methanol (99:1) medium).[33] The fluorescence of **26** was blue shifted upon complexation with Li^+ due to a modulation of the intramolecular charge transfer (ICT) excited state, enabling ratiometric detection of Li^+. The binding stoichiometry was determined as 1:1 host : guest with the binding constant calculated as log K = 2.80. **26** showed a modest selectivity for Li^+ over Na^+ (log $K_{Li,Na}$= -2.4), with no interference from other biologically active cations (K^+, Ca^{2+}, Mg^{2+}) and was stable to changes in pH over the range 3-10. Using a similar receptor

and a boron-dipyrromethene fluorophore, Suzuki and co-workers have developed two ratiometric Li^+ probes **27** and **28** with additional tetramethyl and benzene blocking subunits to facilitate more selective Li^+ binding.[34,35] The indicator dye **27** was covalently immobilized on the surface of porous glass via its hydroxyl group. The resulting glass optode showed dual fluorescence emission response to pseudo-serum containing varying Li^+ concentrations. The obtained signal was maintained in the presence of potentially interfering cations (Na^+, K^+, Mg^{2+} and Ca^{2+}) and across a broad pH range. The sensor response was found to be reversible within the Li^+ concentrations range 10^{-4} M to 10^{-1} M, and showed good repeatability and light stability. The association constant, log K, was estimated to be 2.73. Structural analogue **28** was immobilized to a mesoporous silica thin film and was found to have a higher binding constant for Li^+ (log K) and was responsive between 10^{-6} M to 1 M Li^+ concentration.

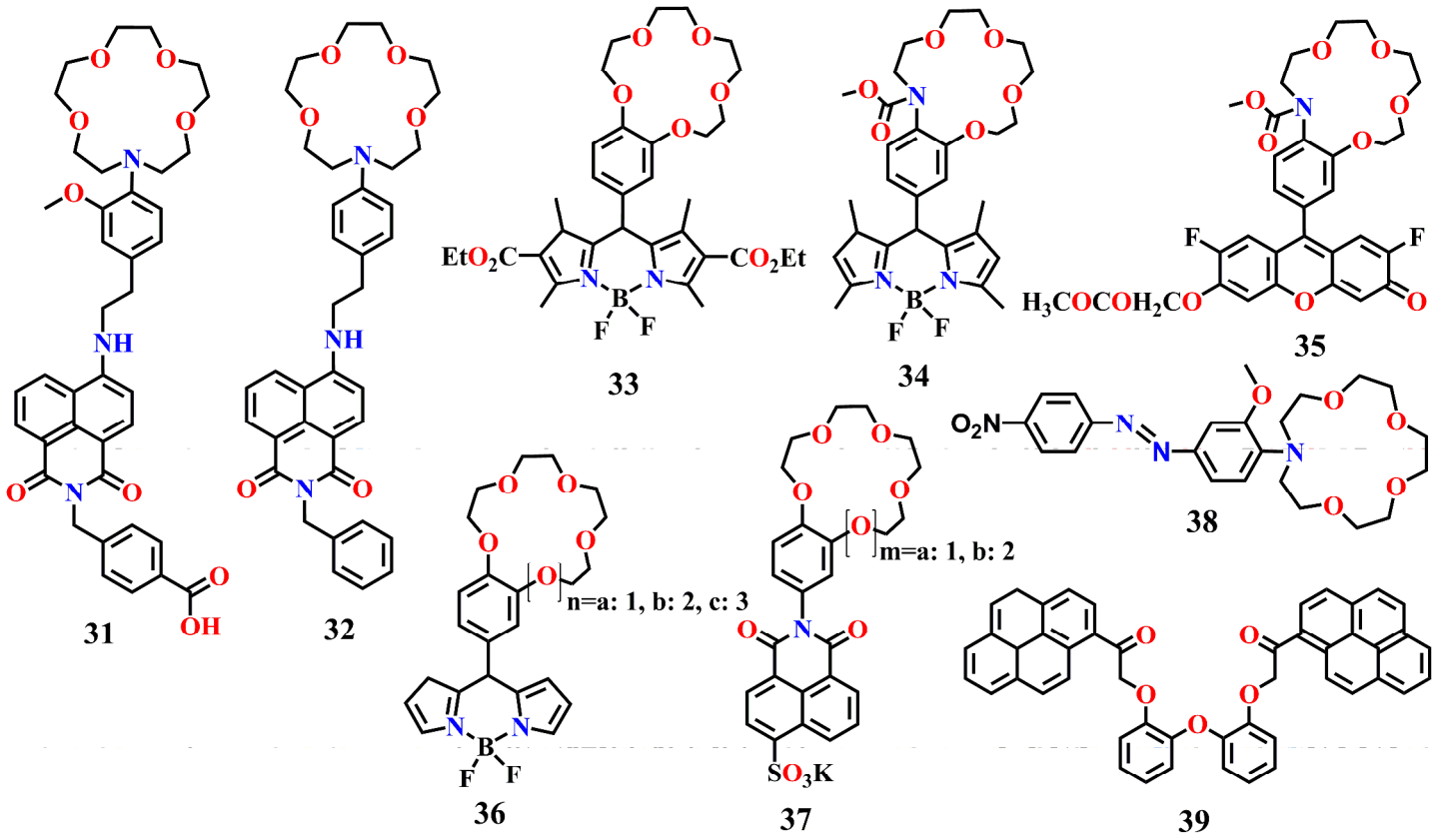


The water soluble (arene)Ru-based 12-metallacrown-3 complex **29** was developed as a selective "Off-On" fluorescent sensor for Li^+ in water (100 mM phosphate buffer, pH 8.0).[36] The high affinity for Li^+, [$K_a(Li^+)$ = 7.4 (± 0.6) 10^2 M^{-1}], over Na^+ and suitability for operation in a complex matrix such as human blood serum are attractive features of this system. However, a major limitation was the small fluorescence enhancement (2.5-fold) upon addition of Li^+. To avoid the complex synthetic steps adopted for the synthesis of the functionalized dihydroxypyridine ligand and to improve the fluorescence quantum yield, an indicator displacement assay (IDA) approach was developed by Severin and coworkers.[33] In this work, the fluorescent dye 8-hydroxy-1,3,6-pyrenetrisulfonate (HPTS) was used as an indicator to interact non-covalently with the reporter **30**[37] resulting in a strong quenching of the fluorescence emission at 510 nm (λ_{exc} = 462 nm) in buffered aqueous solution. The addition of Li^+ ions to **30** : HPTS complex resulted in recovery of the fluorescence signal with an I/I_0 = 6.6. The apparent binding constant was calculated to be 2.3 ± 0.2 x 10^3 M^{-1} with excellent selectivity over competing metal ions such as Na^+ and Mg^{2+}. **30** was also shown to successfully determine Li^+ concentrations in human blood serum. Even though **30** was better than many reported Li^+ sensing systems, a

major limitation was the long response times and potential instability of the metallomacrocyclic receptor to plasma protein.

3.2. Sensors for Na^+ : Since the pioneering work of Minta and Tsein in 1989[38] various probes with binding constants appropriate for the detection of intracellular and plasma levels of Na^+ have been developed.[39] We review the more recent cases here.

PET based probe **31**[40] developed by He and co-workers, comprised a napthalimide fluorophore connected to an N-phenyl azacrown ether via an ethylene spacer for the determination of Na^+. Around the same time, this receptor was also combined with anthracene in a PET format with similarly impressive properties.[41] In addition to the natural increase in oxidation potential offered through chelation of Na^+ by the anilino nitrogen's lone pair, it is enhanced further by the presence of the 2-methoxy subunit that caps the Na^+ corralled in the crown cavity. This capping process requires rotation about the C-N bond resulting in de-conjugation of the amine lone pair and a further increase in oxidation potential of the N-phenyl azacrown ether. This increase in oxidation potential resulted in a remarkable fluorescent enhancement at 540 nm upon Na^+ addition due to the inhibition of PET from the azacrown nitrogen atom. The sensor was successfully immobilized on an amino-functionalized hydrophilic polymer and used to measure plasma Na^+ levels as part of the Opti critical care analyser. This device has been used throughout the world enabling plasma Na^+ levels to be determined within minutes and has no doubt contributed to saving many lives. Optical fibers comprising the same probe (minus the carboxylic acid group for immobilization) **32** were subsequently developed by Englich and co-workers for the determination of Na^+ concentrations in small sample volumes.[42]

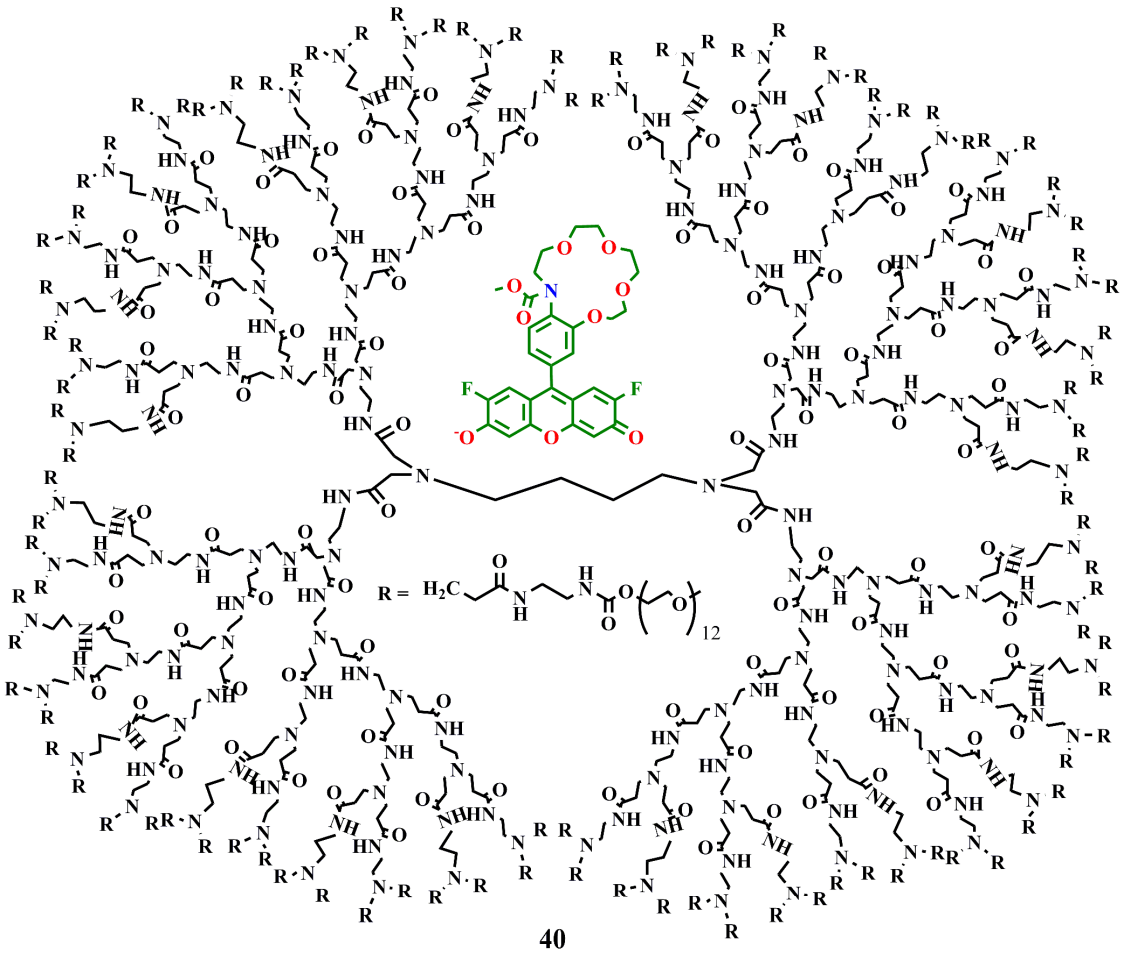


The excellent photo-physical properties of BODIPY as a light-emitting unit has generated many interesting fluorescent sensors for alkali, alkaline-earth, and other metal ions.[43] The BODIPY derivative **33** with 15-benzocrown-5 binding unit showed 'turn-on' fluorescence response due to reducted PET from the benzocrown electron donor to the fluorophore with a remarkable increase in the fluorescence quantum yield by factors of 37 and 7 respectively upon complexation with Na^+ and K^+ in methanol.[44] Similarly, the fluorescence of **34** with an aza-15-benzocrown-5 chelator linked to a BODIPY unit increased 7 and 3-fold upon interaction with Na^+ and K^+, respectively.[45] However, when the fluorophore unit of **34** was replaced by CoroNa Green, the new sensor **35** showed improved Na^+ binding and performed well as an intracellular fluorescent indicator.[45] In a more recent study, BODIPY based probes containing receptors such as 15-benzocrown-5 (**36a**), 18-benzocrown-6 (**36b**) and 21-benzocrown-7 (**36c**) receptors were developed[46] and a direct correlation was found between the size of the crown ether cavity and metal ion selectivity. Sensor **36a** showed a selective fluorescent response for Na^+, **36b** for K^+, while **36c** was selective toward larger sized metal ions such as Ba^{2+}, Cs^+ and Rb^+, with a maximum enhancement for the divalent Ba^{2+}. A similar study comparing the size of the crown ether cavity towards selectivity for alkali metal ions was also performed by Nandhikonda et al, who used 4'-aminobenzo-15-crown-5 (**37a**) and 4'-aminobenzo-18-crown-6 (**37b**) as receptors attached to a 4-sulfo-1,8-naphthalic anhydride fluorophore.[47] The smaller cavity **37a** demonstrated a concentration dependent increase in intensity from 0 to 0.5 mM Na^+ in aqueous solution with no noticeable interference from K^+. **37a** formed a 1:1

binding host-guest complex with an apparent K_d of 1.12 mM. In contrast, the fluorescence intensity of **37b** was linear in the 0 to 6 mM range upon addition of KCl, suitable for monitoring plasma levels of K^+, which range from between 3.5 to 5.3 mM. Chromogenic probe **38**, prepared by Gunlaugssons group, displayed a significant hypsochromic shift in its absorption spectrum upon binding Na^+ with a binding constant (Logβ = 1.25) appropriate for the measurement of Na^+ ion in plasma.[48]

In addition to the PET based sensors discussed above, monomer : excimer based systems have also been designed for sensing Na^+. **39** possesses two pyrene units connected together via a scaffold comprising five oxygen atoms in a structure not dissimilar to a crown ether. In the absence of analyte a strong excimer emission at 524 nm was observed attributed to the intramolecular stacking of pyrene units in the chloroform-acetonitrile (97:3, v/v) solvent. Addition of Na^+ resulted in a decrease of the excimer emission and a concomitant increase in monomer emission at 423 nm. **39** formed a 1:1 complex with Na^+ with a binding constant of 1.14 x 10^4 M^{-1} and showed good selectivity over other physiologically relevant cations.[49]

The use of a polyamidoamine (PAMAM) dendrimer **40**, having sixteen 1,8-naphthalimide fragments in its periphery was developed for the selective fluorescent 'turn-on' sensing of Li^+ in alkaline DMF medium without any interfering effects from the other alkali metal ions such as Na^+ and K.[+50] Following this report, Lamy and coworkers have developed a new Na^+ sensitive nanoprobe (diameter = 6.57± 0.04 nm) by encapsulating a Na^+ dye CoroNa Green (CG) non-covalently in a PAMAM dendrimer (generation 5) nanocontainer carrying a poly(ethyleneglycol) surface (PAMAM-PEG) for imaging neuronal activity in whole brain tissue.[51] The dye/dendrimer ratio of 1.2 was maintained in order to avoid having multiple dye molecules associated with the same dendrimer particle and to avoid self-quenching of the fluorescence. This nanoprobe was found to be very stable, and showed better Na^+ sensitivity (K_d at 81.2 mM) and selectivity than the free CG. It was suggested that this could be due to restricted movements inside the cavity, resulting in reduced non-radiative relaxation and an increased fluorescence lifetime. The PAMAM-PEG-CG system showed good biocompatibility when tested in neuronal cells, was retained for long duration without any alterations of cell functional properties enabling prolonged imaging of intracellular Na^+ dynamics.



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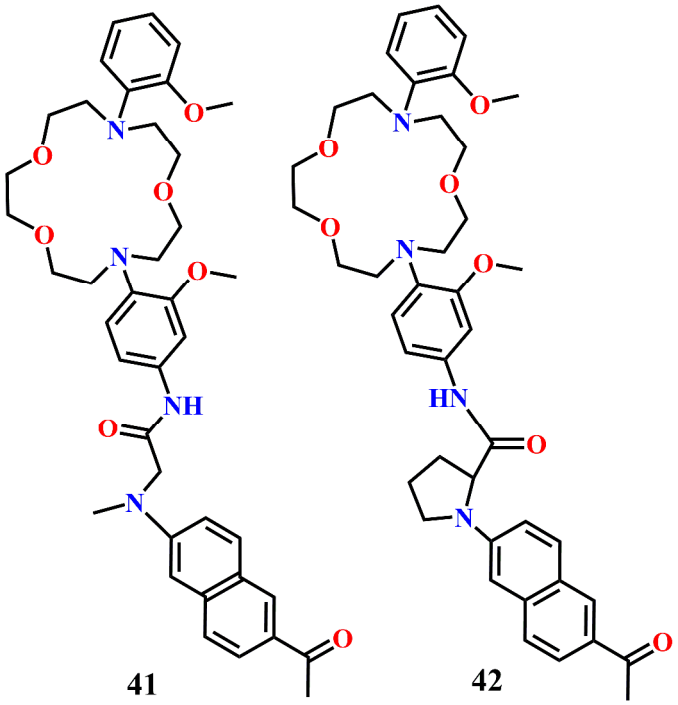
The design of suitable fluorescent systems for live imaging is quite challenging due to many requirements such as safe delivery to cells, selectivity and sensitivity to target ions an excitation wavelength in visible or near infrared region, affinity under wide pH range, absence of cytotoxicity, targeting to the appropriate subcellular compartment, absence of cell leakage or photobleaching, absence of interaction with cellular physiology and high brightness with large fluorescence changes during imaging. Therefore, considerable effort has been devoted to developing fluorescent sensors based on one-photon and two-photon excited fluorescence (TPEF).[52] The cellular imaging applications of commercially available single-photon sensors like sodium-binding benzofuran isophthalate (SBFI-AM) and Sodium Green (SG-AM) acetoxymethyl esters suffer from a short excitation wavelength (< 500 nm), limiting their potential use in intact tissue imaging because of the limited penetration of light at this wavelength through mammalian tissue. Using two-photon excitation schemes enables excitation of fluorophores using two low energy photons (i.e. 2 x 800nm) that essentially add together to form one higher energy photon (1 x 400 nm), provided they are delivered on the femtosecond time scale. The advantage of such an approach is that mammalian tissue has maximum transparency to light in the 750-900 nm range, the so-called photo-therapeutic window. Indeed the penetration of light at 800nm is some four times greater than at 600 nm. However, not all fluorophores have sufficient two-

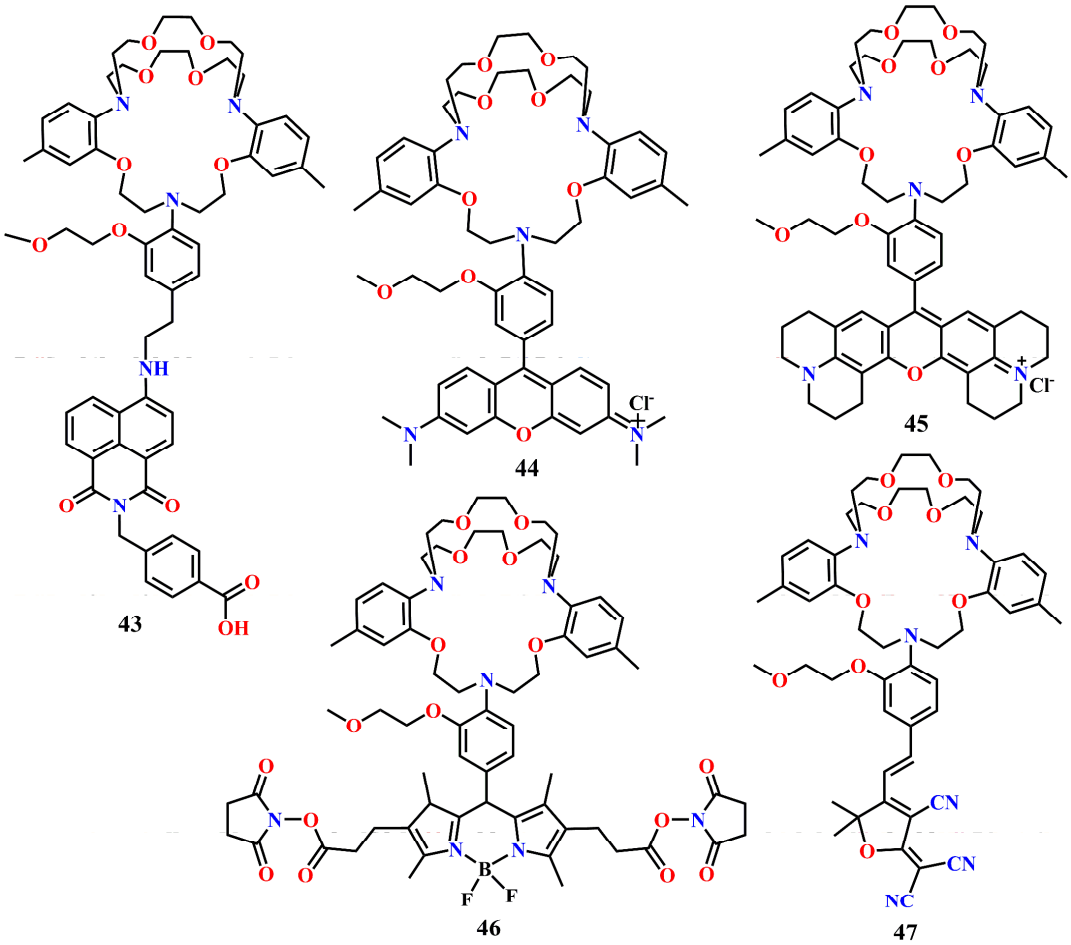
photon absorption cross sections that are suitable for effective two-photon excitation. Probe **41** comprising the known Na^+ receptor 1,7-diaza-15-crown-5' connected to an acedan fluorophore was tested for its ability to function as a TPE Na^+ sensor.[53] When complexed with Na^+, **41** exhibited two-photon absorption cross-section of 95 Goeppert Mayer units (GM) at 780 nm, greater than the commercially available sensors SG-AM and SBFI-AM. **41** was used to image the change in Na^+ levels in astrocytes upon addition of Ouabain, a steroid hormone that increases the cytosolic free Na^+ concentration by inhibiting Na^+/K^+-ATPase and glutamate. **41** was easily loaded into the cells and selectively detects intracellular free Na^+ in live cells and living tissues at depths of between 100–200 μm for extended time periods. However, **41** showed a modest FE (8-fold) in the presence of Na^+. Therefore, in order to develop a more sensitive two-photon sensor for intracellular Na^+, **38** was modified by using a prolinamide as a linker between the receptor and the acedan fluorophore.[54] Under physiological conditions (10 mM MOPS, pH 7.0), the resulting probe **42** displayed absorption and emission maxima at 366 nm and 500 nm respectively. Upon complexation with Na^+, the fluorescence intensity of **42** was increased dramatically due to the blocking of the PET process. An improved FE of 15 for **42** was obtained and the probe showed good selectivity over K^+ and good operability over a broad pH range. The dissociation constant (K_d) values for **42** and Na^+ measured in the absence and presence of 135 mM K^+ were 18 and 22 mM, respectively. The values are well within the range of intracellular Na^+ in live cells. This probe can be easily loaded into the cell to monitor the fluctuation of intracellular Na^+ in living tissue at 90–180 mm depths for a relatively long time period without interference from other biologically relevant metal ions using two-photon excited microscopy (TPM).



3.3. Sensors for K^+ : As was the case with Na^+ sensors, the main challenge in developing selective K^+ sensors is potential interference from other metal ions particularly Na^+. This has particular relevance in biological media where the Na^+ concentration can be significantly higher than K^+ concentration (i.e. Na^+/K^+ 30 in human blood serum). Like our opening example for Na^+, we open this section with a K^+ specific probe that also found commercial exploitation in the Opti-critical care analyzer. Following a modular approach, He and co-workers retained the same napthalimide fluorophore and ethylene spacer as used in the Na^+ probe **31**, but substituted the N-phenyl azacrown receptor with the larger cavity containing tri-azacryptand (TAC) as a receptor for K^+. [55] The probe **43**, was selective for K^+ over Na^+ and sensitive for K^+ in the physiological range. This is an excellent example of the simplicity offered by the PET design principle where the fluorophore and spacer units remain unchanged while the receptor unit can be varied to meet the required demands of selectivity and sensitivity. Unfortunately, while extremely successful for analyses where *in situ* calibration is not necessary, such as in the Opti-critical care analyser, single wavelength PET probes struggle to compete with ratiometric probes when internal calibration is a pre-requesite. Such probes are based predominately on internal charge-transfer (ICT) type mechanisms. In probe **44**, the TAC receptor was retained and directly connected to a xanthylium fluorophore without a spacer unit in an ICT format.[56] In 5 mM HEPES at pH = 7.04, the fluorescence of **44** (7 μM) was increased up to 14-fold in the presence of 50 mM K^+ with K^+-to-Na^+ selectivity >30. The K^+ waves in brain space were effectively visualized using **44**, which opened the door for the development new sensing systems **45** to **50** using TAC. The binding kinetics of K^+ with **44** and newly developed sensor **45**[57] (TAC-crimson) was reported by Verkman and coworkers. Similar to **44**, probe **45** also showed a fluorescent enhancement (12-fold) at 597 nm upon increasing the K^+ concentration from 0 to 200 mM. **45** could operate effectively between pH 6-8 and was insensitive to both Na^+ and Li^+. The rapid millisecond K^+ binding kinetics with **44** and **45** demonstrated their utility for measuring changes in K^+ concentration during rapid neural signaling and ion channel gating.

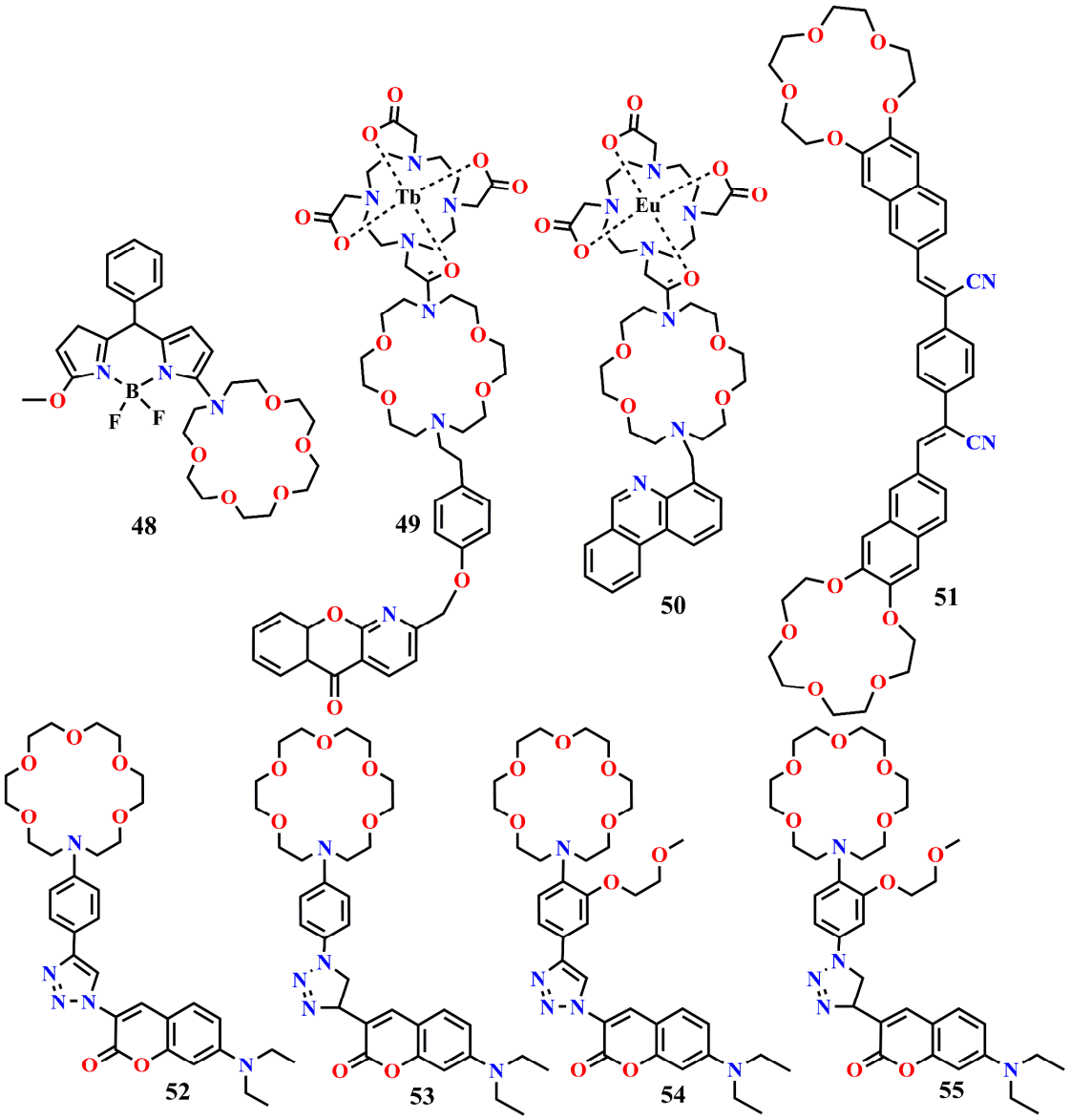
Macromolecular probe **46** (TAC-$Lime_{dex}$) comprising a TAC receptor directly connected to a BODIPY fluorophore and grafted onto a dextan polymeric backbone was developed to determine extracellular K^+ levels during plasma membrane K^+ transport due to its membrane impermeability.[58a] This system was further optimized by attachment of a red emitting tetramethylrhodamine (TMR) reference fluorochrome for the ratiometric fluorescent measurement of extracellular K^+ in airway surface liquid (ASL).[58b] ASL is the thin fluid layer lining airway surface epithelial cells, whose volume and composition are tightly regulated and may be abnormal in cystic fibrosis (CF). The TMR derivative of **46** was K^+ selective and showed a >4-fold enhancement with increasing K^+ concentration, between 0 and 40 mM. This study revealed the involvement of apical and basolateral membrane ion transporters in maintaining a high ASL.

The intracellular K^+ probe **47** was constructed using the TAC receptor connected to the strongly electron withdrawing fluorophore 2-dicyanomethylene-3-cyano-4,5,5-trimethyl-2,5-dihydrofuran (TCF) in an ICT format.[59] The push-pull nature of **47**, where the electron density of the N-phenyl atom is markedly reduced due to the strongly electron withdrawing fluorophore, weakens its binding to K^+ increasing its K_d value to 88 mM suitable for intracellular measurements. Indeed the authors demonstrated the ability of **47** to measure intracellular K^+ levels before and after addition of K^+ efflux stimulators. However, despite the strong ICT nature of **7**, the Off-On FE occurred at a single wavelength (650 nm) meaning ratiometric analysis was not possible.



Combining aza-18-crown-6 as K^+ receptor with BODIPY as fluorophore in an ICT format to produce probe **48** enabled ratiometric detection of K^+ over the 0-8.9 mM range in acetonitrile solvent.[60] Among the tested metal ions (Li^+, Na^+, K^+ and Cs^+), **48** displayed a significant blue shift in absorption and emission upon K^+ binding. Molecular modelling studies suggested a considerable conformal change occurred upon K^+ binding that involved participation from the aza-crown ether. Lanthanide complex **49** containing a Tb^{3+} functionalsied azo- crown ether receptor and azaxanthone antennae was developed for the time-gated luminescence detection of K.[+61] Lanthanide complexes have significantly longer lifetimes than endogenous fluorescent compounds and thus can reduce problems associated with autofluorescence in time gated experiments. **49** showed high

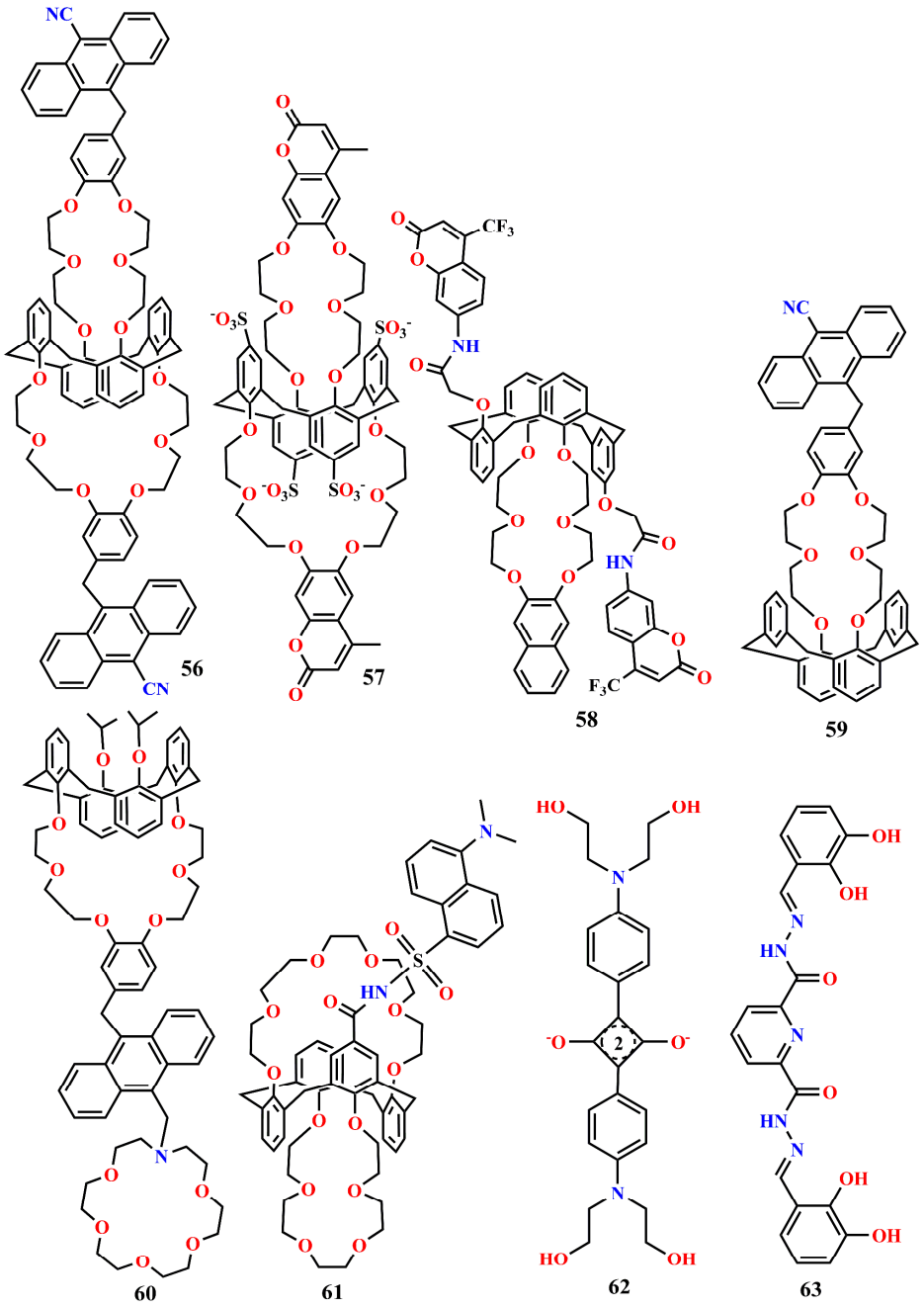
selectivity with a 22-fold increase in luminescence intensity between 0 and 10 mM K^+. The encapsulation of K^+ in the crown ether cavity required participation from the aryl ether, resulting in a conformation change that brought the antennae closer to the Tb^{3+} facilitating its sensitized excitation. In addition, **49** showed a high selectivity for K^+ over Na^+ (93-fold), which was better than the previously reported Tb-based sensors (5 to 10-fold).[62] However, its poor aqueous solubility limited its use in biological environments. In contrast, Gunnlaugsson's europium complex **50** showed excellent solubility in water, possessed a high affinity for K^+ in the 0-10 mM range and displayed good selectivity over the physiologically relevant Na^+, Ca^{2+}, Mg^{2+} and Li^+.[63] The long luminescence lifetime (>1 ms) of **50** readily enables time-gated imaging. Also, the large Stokes shift of **50** (λ_{exc} = 267 nm, λ_{em} = 593 nm) limit any self-absorption problems at high concentration due to the negligible overlap between the absorption and emission spectra, making this system ideal for imaging extra-neuronal K^+ fluxes during an action potential.



The bis(crown ether) system **51** was inspired by the higher K^+ extractability of naphtha-15-crown-5 compared to benzo-15-crown-5.[64] The fluorescence of **51** was enhanced due to the formation of sandwich type complex with K^+ and displayed excellent K^+ selectivity over Na^+. Recently, the crown ether based K^+ selective fluorescent sensors **52-55** were developed by the π-conjugation of N-phenylaza-18-crown-6 ether and 7-diethylaminocoumarin.[65,66] In CH_3CN, **52** showed a significant fluorescence enhancement upon addition of Na^+ (FEF = 58) and K^+ (FEF = 27). The constitutional isomer **53** showed similar selectivity but the FEFs for Na^+ (FEF = 17) and K^+ (FEF = 15) were significantly lower than **52** due to poor planarity. Importantly, under simulated physiological conditions (10 mm Tris buffer, pH 7.2), the fluorescence of **53** was enhanced in the presence of 160 mM K^+ with a FEF of 2.5 and a dissociation constant K_d of 260 mM. Interestingly, no noticeable fluorescence change was observed with Na^+ because of its high hydration enthalpy. Furthermore, in order to decrease the K_d value of **53**, probes **54** and **55** were designed by introducing a 2-methoxyethoxy lariat group to the phenylaza-[18]-crown-6 receptor.[66] The stabilities of **54** and **55** were about tenfold higher than **53** because of the lariat group which provides a seventh donor atom to complex with K^+. Probe **54** ($\Phi = 0.062$, $\lambda em = 493$ nm) showed a 2.5-fold fluorescent enhancement in the presence of 100 mM K^+ ions with the quantum yield increasing to 0.184 (Much bigger than 2.5 fold-check) under simulated physiological conditions. **54** was subsequently incorporated within a polyacrylamide-co-polyacrylonitrile hydrogel, which enabled the continuous monitoring of physiological K^+ levels with a rapid response time.

3.4. Sensors for Cs^+

The heavier alkali metal ion Cs^+ is not an essential element but its role in biochemistry and physiology is well established.[67] Cs^+ ions activate the Na/K-dependent ATPase in the absence of K^+ and are transported into cells. The inadvertent consumption of radioactive Cs^+ generated from fission reactions can induce serious toxicological problems. The negative health effects of Cs^+ include cardiovascular disease and gastrointestinal distress.[68] Therefore, many fluorescent based sensing systems have been developed for the detection of Cs^+ mainly by employment of calixarene and calixcrown macrocyclic receptors incorporating fluorophores such as coumarin, anthracene, naphthalene etc.



The fluorescent PET sensor based on *1,3-alternate* calix[4]arene **56** containing two crown moieties was developed for sensing Cs^+ in CH_2Cl_2/CH_3OH (1:1, v/v).[69] The weak fluorescence of **56** was enhanced 11-fold upon complexation of Cs^+ by the ether oxygen atoms inhibiting the PET process. In an attempt to develop a more water soluble fluorescent system for Cs^+, Valeur and coworkers used tetrasulfonated calixarene **57** again with two crown-6 units as receptor.[70] Upon complexation with Cs^+, a significant fluorescent enhancement of **57** was observed due to the increased donor ability of the coumarin units. **57** showed no interference from other alkali metal ions and was operable over a wide pH range of 6-9. Subsequently, **58**, a calix[4]arene bearing one 2,3-naphthocrown-6 and two coumarin amide units at the lower rim in *partial-cone* conformation was reported for the fluorescent sensing of Cs^+ by a FRET-based mechanism,[71] with the naphthalene unit functioning as an

energy donor and the nearby coumarin unit as acceptor. Binding of Cs^+ to the crown cavity reduced PET to the napthalimide enabling a more efficient FRET process to the coumarin unit. While not effective in aqueous medium, this probe also demonstrated the ability to sense F^- ion offering possibilities for molecular logic.

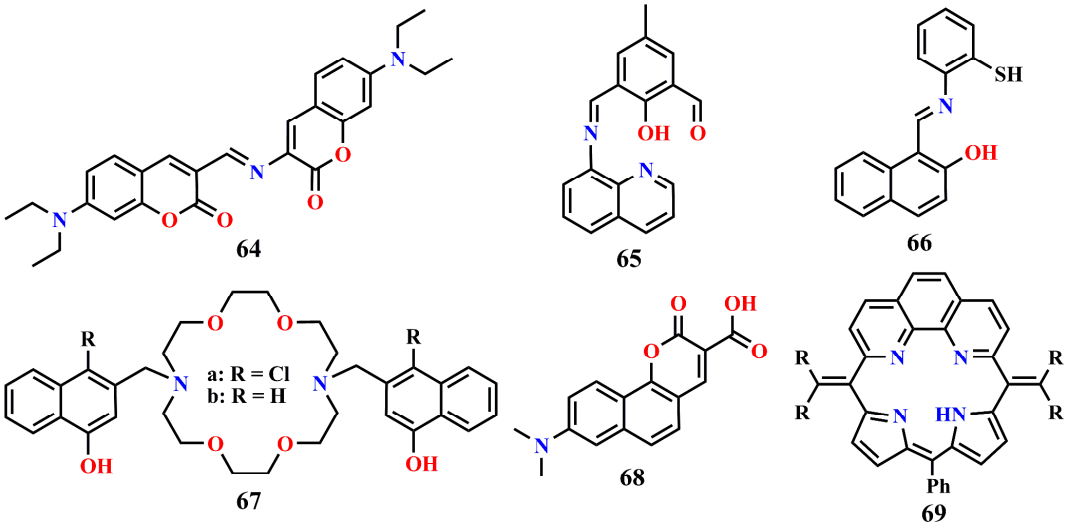
Single fluorophore systems with calix[4]arene type receptors have also been developed as Cs^+ sensors. **59**, structurally analogous to **56**, showed both increased selectivity towards Cs^+ and also remarkable fluorescent enhancement (20-fold) in CH_2Cl_2/CH_3OH (1:1, v/v).[72] Also, the fluorescent quantum yield of the **59**.Cs^+ complex was 54 times greater than the uncomplexed **59**. In an extension to this approach, the same group combined calixarene and azacrown ether units at opposite ends of a common anthracene fluorophore. In basic methanolic solvent, a PET process from the N atom of the azacrown ring of **60** resulted in the weak emission.[73] When the N atom was protonated in acidic medium, the addition of Cs^+ ion resulted in a 3.8-fold FE due to a cancellation of the PET process. However, in basic medium, the system demonstrated high selectivity for K^+ with a 6.4-fold FE. Subsequently, the fluorogenic sensor **61**, based on 1,3-*alternate* calix[4]arenebis-(crown-6) containing a dansyl fluorophore was reported for sensing of Cs^+ in acidic conditions (pH = 3.5) in MeCN-H_2O (1:1, v/v) solution.[74] The fluorescence of **61** was gradually enhanced accompanied by a hypsochromic shift with a LOD of 4 x 10^{-7} M.

Recently, a squaraine dye comprising dihydroxyethanolamine moieties as binding unit (**62)** was reported for the highly sensitive (LOD = 0.096 μM) and selective (limited response to other metal ions) detection of Cs^+ ion in aqueous medium.[75] The efficient quenching of the squaraine unit to 7.6% of its initial value clearly delineated the applicability of **62** for Cs^+ monitoring using an On-Off fluorescence response. Another highly selective probe with a nanomolar detection limit with Off-On fluorescence response **63** was reported for the detection of Cs^+ in aqueous medium.[76] In this approach, fluorescent organic nanoparticles (FONs) of **63** were prepared by a re-precipitation method. **63** showed an aggregation-induced emission (AIE) at 412 nm which was remained stable in the pH range 4-9. The emission band of the FONs was remarkably enhanced at 412 nm due to the inhibition of PET upon formation of the **63**.Cs^+ complex. The detection limit for this system was found to be 70 nM for Cs^+ ions. Also, the detection of Cs^+ by **63** was not influenced by many other tested metal ions including alkali and alkaline earth metal ions.

4. Alkaline-earth metal cations

4.1. Sensors for Mg^{2+}: Magnesium is the second most abundant divalent cation present in intracellular fluid and plays a significant role in activating more than 300 different enzymes. The functioning of cells and level of electrolytes in the body are dependent on magnesium. It regulates neuronal activity, blood pressure and cardiac

excitability.[77] Hypomagnesaemia can be induced by prolonged fasting, alcoholism, and surgical stress while hypermagnesaemia can lead to coma and death. The use of magnesium sulfate in the treatment of vasospasm may be iatrogenic if it leads to hypermagnesaemia.[78] Therefore, due to important role of Mg^{2+} in controlling proper physiological functioning has led to significant interest in the development of effective probes for its measurement.



Bharadwaj *et al*. synthesized probe **64** comprising two coumarin units connected via an imine bond following a Schiff base condensation reaction.[79] The incorporation of a C=N group to control emission from an attached fluorophore has been used by several different groups when designing fluorescent probes for metal ions. Usually, the C=N unit is attached directly to the fluorophore component which may also contain other atoms involved in binding the target ion. Other chelating groups, positioned at the opposite end of the C=N unit to the fluorophore also contribute to analyte binding. In the absence of the target species, excited state deactivation occurs predominately through a C=N isomerization process. Binding of the target analyte helps lock the C=N unit in place reducing non-radiative decay brought about by the isomerization process. The outcome is an Off-On fluorescent response to the target analyte. Such a strategy is apparent in **64**, with the two lactone carbonyl oxygen atoms and the imine nitrogen atom used to bind the Mg^{2+} ion. In the absence of Mg^{2+}, **64** displayed a strong absorption band at 488 nm with only weak emission. Upon addition of Mg^{2+} ion, the absorption band was red shifted to 555 nm with a 550- fold in emission centered at 600 nm. **64** showed good selectivity over other divalent cations such as Ca^{2+} or Zn^{2+} but its inability to operate in aqueous medium has obvious drawbacks. The diformyl-*p*- cresol-8-aminoquinoline (**62**) probe developed by Ali *et al* exhibited fluorogenic recognition of Mg^{2+} in semi-aqueous medium (CH_3CN / HEPES pH=7.2 9:1,v/v).[80] Upon addition of Mg^{2+} ion to a solution of **65**, a significant enhancement in fluorescence intensity was observed that was accompanied with a gradual blue shift from 562 nm (unbound) to 526 nm (bound). **65** displayed good biocompatibility and was used to monitor Mg^{2+} concentrations in HepG2 cells.

Singh *et al*. developed the Schiff base probe **66** using a facile reaction between the commercially available 2-aminothiophenol and 2-hydroxy-1-naphthaldehyde in methanol.[81] Excitation of **66** facilitates an excited state intramolecular proton transfer (ESIPT) process converting the keto form to its enol tautomer, which showed selective recognition of Mg^{2+} ion in a THF/H_2O (9:1,v/v) solvent system. Binding of Mg^{2+} to the enol form of **66** was accompanied by the formation of a red-shifted band at 427 nm with a concomitant reduction in the band associated with the free enol form. This enabled the ratiometric detection of Mg^{2+} with excellent selectivity against physiological and environmentally relevant metal ions. Again, the FE observed upon the binding of **66** with Mg^{2+} was attributed to the inhibition of the C=N isomerization process. Analysis of the UV-Vis absorption spectra of **66** before and after addition of Mg^{2+} ion revealed that Mg^{2+} did not bind in the ground state.

In an alternative approach, Prodi *et al*. reported the development of **67a** in which a diazo-18-crown-6 was linked to two 5-chloro-8-hydroxyquinoline-7-yl (CHQ) groups via the diazocrown N atoms in a PET type format.[82] In addition to PET, HQ derivatives also experience an intermolecular photoinduced proton transfer (PPT) process between the hydroxyl group (which is a relatively strong photoacid) and the nearby quinoline nitrogen (which in turn, is a strong photo base). A pH titration methanol-water (1:1, v/v) solution revealed the amino nitrogen had a pK_a value 2.8 while the phenolic group was 10.2. Complexation of Mg^{2+} with **67a,** lowered the pKa of the phenolic group attributed to involvement of the phenolic group in the binding process increasing the acidity of the phenolic proton. In the absence of Mg^{2+} **67a**'s emission band was centered at 540 nm which remained unchanged over a broad pH range (2-13). Upon addition of Mg^{2+} a huge FE of 1000 was observed due to the cancellation of both the PET and PPT processes.

In a subsequent study, Prodi et al used **67a** and its derivative **67b** for the measurement of Mg^{2+} in living cells.[83] The absorption and emission profiles of both **67a**-**b** recorded in Dulbecco's phosphate- buffered saline (DPBS), displayed an absorbance band between 230-250 nm with the emission centered at 500 nm. Upon addition of Mg^{2+} a bathochromic shift was observed in the absorbance spectrum accompanied by a significant enhancement the fluorescence spectrum, again due to inhibition of the PET and PPT processes. Moreover, the sensor displayed good selectivity over competing ions such as Ca^{2+}, Mn^{2}, Zn^{2+} ions. These HQ derivatives proved effective at measuring Mg^{2+} concentration using TPE in living HC11 cells when the intracellular ion concentration was modulated using carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP).

Probe **68** was designed by Cho and coworkers[84] for the selective recognition of free Mg^{2+} ions in the living Hep3B cells and in live tissues using two-photon excitation. The metal complex exhibited an emission band at 559 nm upon single photon excitation at 443 nm. Upon addition of Mg^{2+} ion to a solution of **68**, a significant enhancement in the intensity at 559 nm was observed that was not evident for the other cations. This FE upon addition of Mg^{2+} was also evident when **68** was excited using TPE excitation at 880 nm. The probes

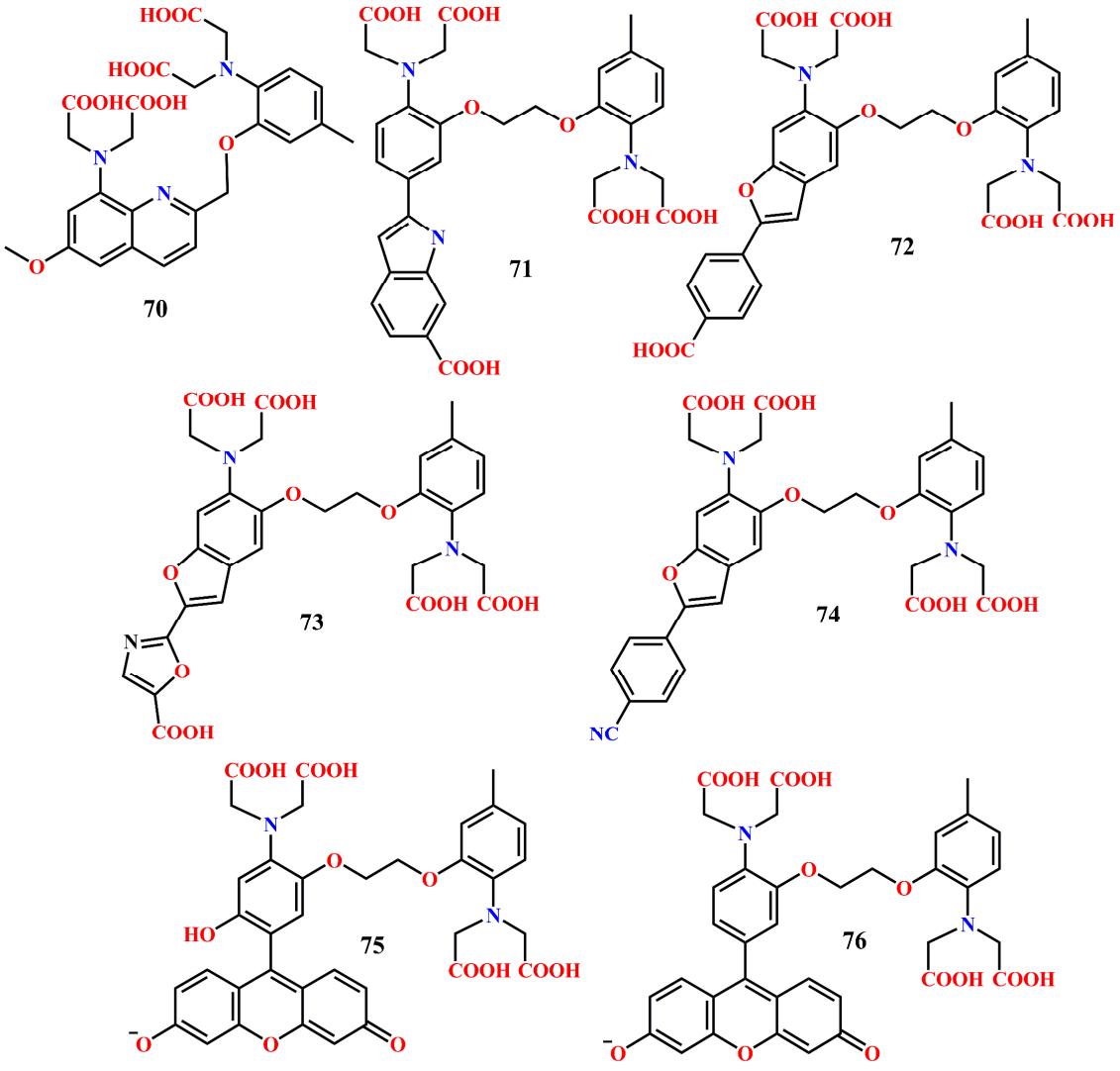
proved effective at detecting Mg^{2+} concentration in living cells without interference from Ca^{2+} ions and was also effective at imaging endogenous stores of free Mg^{2+} in live tissues.

Naruta *et al*. had developed a novel porphyrin analogue **69** with an embedded 1, 10- phenanthroline moiety.[85] **69** had a gable-type nonplanar macrocyclic framework and the inner cavity was fabricated with a small monoanionic coordination sphere, which it suitable for complexation with Mg^{2+} ion. **69** displayed a weak emission at 572 nm in acetonitrile and fluorescence band at 583 nm in HEPES buffer and DMSO solution (7:3, v/v). Addition of Mg^{2+} ion enable generated a new red-shifted peak enabling ratiometric detection.

4.2. Sensors for Ca^{2+}: Calcium is an essential nutrient and is the most abundant element in the human body.[86] In fact it is the most common of the mineral ions in the body,[87] accounting for approximately 2% of total body weight.[88] It is of vital importance to both the skeletal and soft tissues being involved in a range of bodily processes such as bone mineralization, blood coagulation, muscle contraction, regulation of nerve excitation and cell growth and differentiation.[89] Abnormally high or low plasma levels of calcium are known as hyper-and hypocalcaemia respectively. Hypercalcaemia is a potentially life-threatening abnormality that can be difficult to diagnose and indeed is commonly misdiagnosed.[90] It presents in primary and secondary care and has shown a prevalence as high as 18% in hospital patients.[91] Hypocalcaemia usually presents in people with cancer [92] and it is frequently associated with cancers of the breast, lung, head, neck and kidney. In addition to this, abnormalities in calcium-signaling can result in neurodegeneration, [93] heart disease, [94] skeletal muscle defects, [95] and disorders of the central nervous system.[96] Therefore, the need for monitoring intracellular and plasma calcium levels is extremely important.

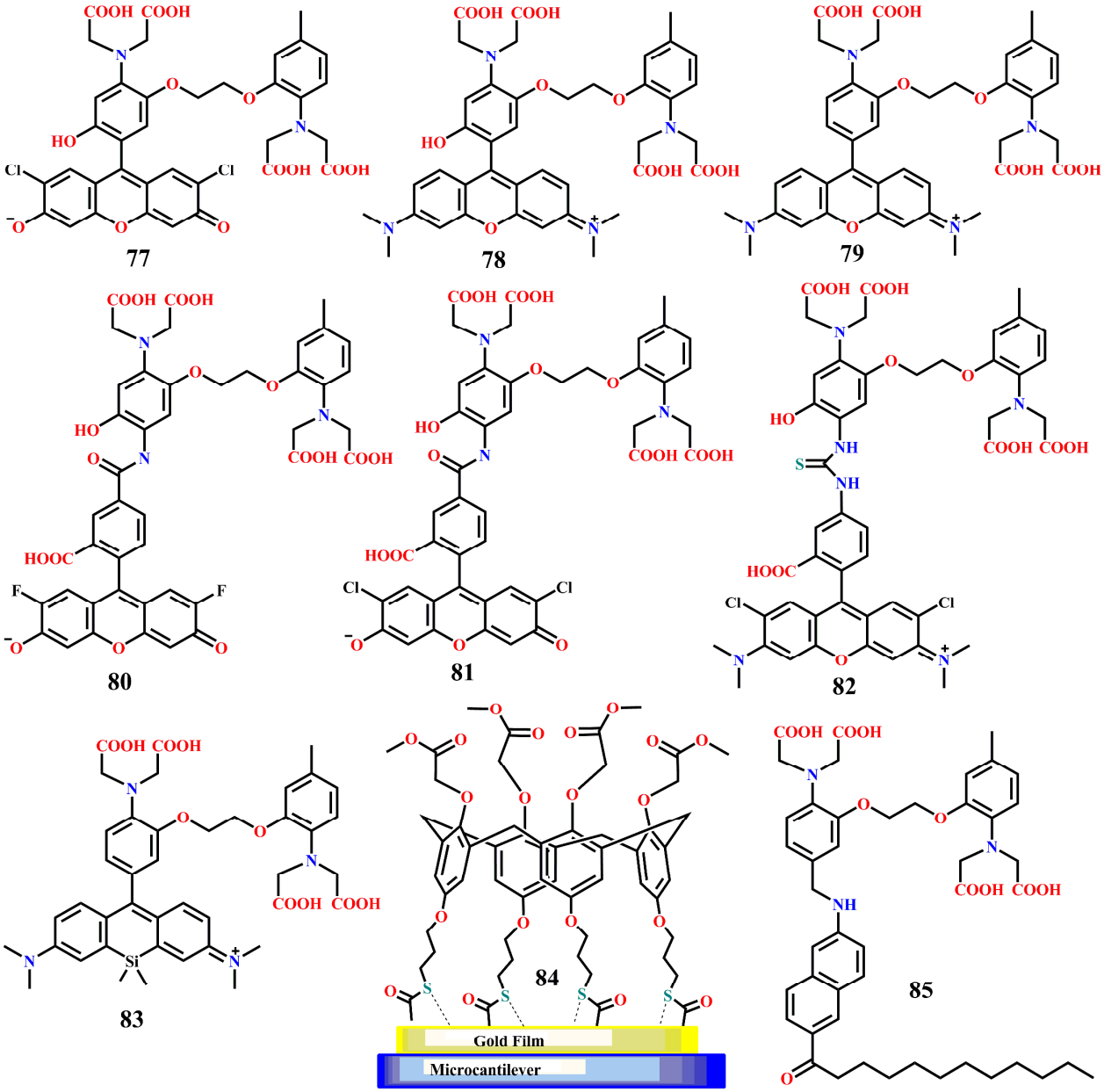
Our understanding of these ubiquitous roles of calcium has been, in part, rapidly advanced by the development of fluorescent indictors.[97] Due to the abundance of calcium in the body and the fact that it is present in numerous cell types at various concentrations, a variety of florescent calcium indicators have been developed over the past 30 years. The first fluorescent probes for calcium were reported[98] in the 1980's by Nobel laureate Roger Tsien and co-workers. The majority of Ca^{2+} probes generated by this group contain the same receptor unit, 'BAPTA' (1, 2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid) chosen due to its similarity in structure to the calcium chelator EGTA (ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid). The modification by Tsien and coworkers improved the chelator by altering its pKa and increasing selectivity over Mg^{2+}, giving a receptor unit which is selective, sensitive toward calcium and capable of operating at physiological pH. Their first work involved the development of 'quin-2' **70** which was based on a quinolone fluorophore linked to a benzene ring via a monoether spacer, with an iminodiacetate receptor on both the quinolone and benzene units to bind Ca(II) ions.[98] It was used as a membrane-permeable ester-derivative which was subsequently cleaved by hydrolysis following cellular uptake, leaving the quin-2 tetra-

anion which became trapped in the cytosol. Ca(II) binding resulted in increased fluorescence again via cancellation of the PET mechanism. However, this sensor had several perceived limitations such as; a near UV excitation wavelength leading to problems with autofluorescence, low quantum yield and a relatively poor selectivity for calcium.[99] Further improvements led to the indo-1, fura-1, 2 and 3 (**71-74**),[100] fluo-1,2 and 3 (**75-77**), and rhod-1 and 2 (**78**, **79**) [101] probes which had improved and varied characteristics compared to **70**. Of these, fura-2 **73** is still one of the most successful and popular Ca(II) indicators and remains the standard for quantitative intracellular Ca(II) measurements.



73 is formed from a stilbene fluorophore linked to a tetracarboxylate receptor unit in the form of two separate dicarboxylate sites via a tertiary amine. These carboxylate groups chelate calcium with 1:1 stoichiometry causing an enhancement in fluorescence. **73** exhibits properties associated with ICT- and PET-based systems, in that binding of calcium ions causes a hypsochromic shift in the excitation maximum from 363nm to 335nm[102] as well as an increase in emission at 510nm when excited at 335nm due to the cancellation of the PET mechanism. Either method can be used for the quantification of calcium levels, however **73** has

found most use as a stand-alone ratiometric probe for intracellular calcium levels,[103,104] whereby the ratio of the excitation spectra at 340 and 380nm, when obtained at 510nm is used to quantify Ca(II) concentrations.



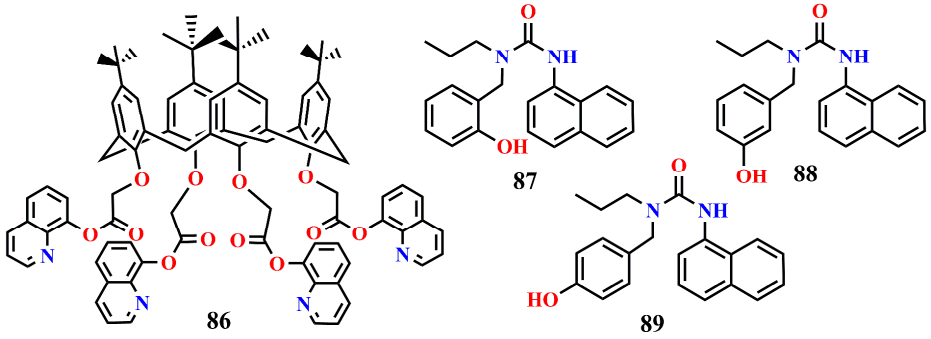
Other, more recent developments in this field have led to novel probes such as Oregon green BAPTA **80**, calcium green **81**, calcium orange [102] and 'CaSiR-1'[105] **83** to name a few. An extensive list of calcium probes can be found in 'The molecular probes handbook.'[102]

An alternative receptor for Ca^{2+} chelation was developed by Georghiou *et al* who prepared a thioacetate substituted calix[4]arene (**84**) for use in aqueous medium.[106] The upper rim of calixarene was functionalized with thiacetate groups, which enabled immobilization of the probe on a gold film. The lower rim of calixarene was functionalized with methyl esters, which formed selective cavity for Ca^{2+} ion. STM and TEM images

confirmed immobilization and the probe showed high selectivity and sensitivity (LOD = 10^{-11} M) for Ca^{2+}. Cho e*t al* have developed **85** with a long hydrophobic tail to visualize near membrane Ca^{2+} levels in living cells.[107] Two photon excitation of **85** resulted in an emission that enhanced significantly upon addition of Ca^{2+} due to a cancellation of the PET process. The fluorescence from **85** showed minimal photobleaching and was capable of monitoring calcium waves at a depth of about 150 mm living tissues.

4.3. Sensors for Sr^{2+}: Although Strontium lacks the physiological relevance of its Group II colleagues Mg^{2+} and Ca^{2+}, it has found many commercial uses from its presence in certain alloys, paint pigments, ferrite magnets and fluorescence lights.[108,109] However, perhaps understandably, its detection has not received the same level of interest as the other Group II cations discussed so far.

Menon et al. developed a Sr^{2+} probe using a calix[4]arene based receptor **86**[110] with quinolone units on the lower rim. In an acetonitrile based solvent system addition of Sr^{2+} to **86** resulted in an enhancement of fluorescence due to a cancellation of PET with a LoD =1.04. In addition, binding of Sr^{2+} ion by **86** led to a bathochromic shift in the absorbance spectrum and 1:1 binding stoichiometry was confirmed by mass spectroscopy. The performance of **86** was unaffected over a broad pH range and proved capable of detecting Sr^{2+} in waste water samples.

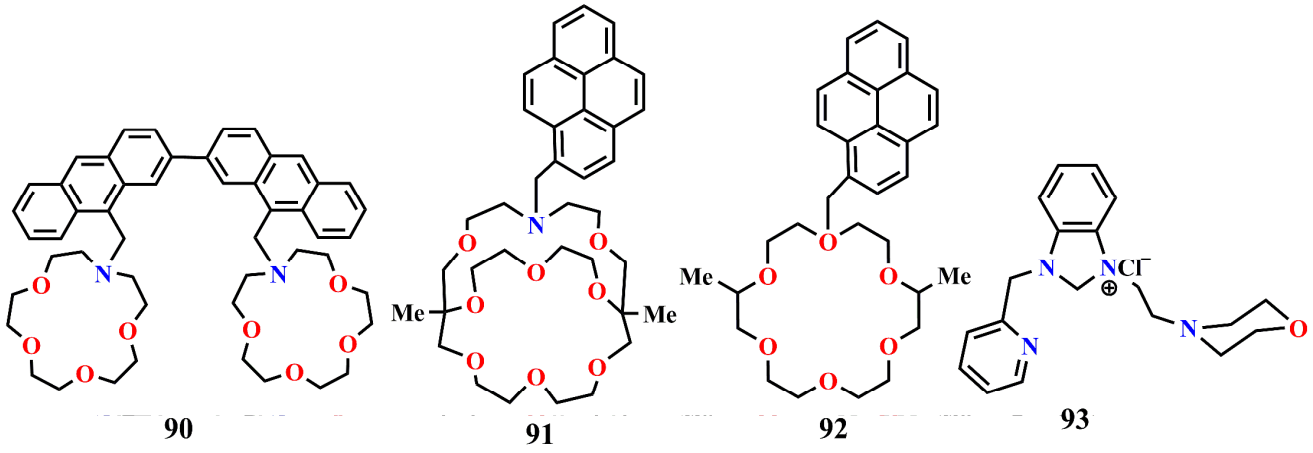


Singh *et al*. encapsulated naphthalene-phenol hybrids **87-89** with organic nanoparticles (ONs) for the selective detection of Sr^{2+} in aqueous medium.[111] The facile preparation of these ONs was a contributing factor in their choice by the authors. Following a re-precipitation method that included the injection of a small volume of **87-89** into a large volume of a non-solvent, stable ONs were produced.[112] Selectivity studies demonstrated ONs comprising all three probes were selective for strontium ion via and enhancement in fluorescence intensity with the lowest LOD =184 μM observed for ONs comprising **86**. The loaded ONs were capable of determining the level of Sr^{2+} in oral healthcare products.

4.4. Sensors for Barium^{2+}: Like the earlier alkaline earth metals discussed above, Ba^{2+} also plays an important role in biological systems. Elevated levels of Ba^{2+} in humans can cause acute gastroenteritis, loss of deep

reflexes, muscular paralysis, respiratory failure, or even death.[113] Use of Ba^{2+} in a number of industries such as the paint, glass and fireworks etc. has provided a pathway for its introduction to the environment.

Kondo and Unno *et al.* derivatized 9,9'-dimethyl-2,2'-bisanthracene with two aza-15-crown-5 receptors in a PET format to form probe **90**[114] for the fluorescent detection of Ba^{2+}. In a CH_3CN/H_2O (98/2, v/v) solvent system, addition of Ba^{2+} to **90** resulted in the appearance of blue fluorescence centered at 472 nm with good selectivity over other Gp II metal ions. The enhanced fluorescence upon binding Ba^{2+} was attributed to the formation of intermolecular and possibly intramolecular sandwich complexes between **90** and Ba^{2+} with a corresponding cancellation of the PET process.



90 **91** **92** **93**

Nakatsuji and Akashi *et al.*[115a] developed pyrene functionalized monoazacryptand probe **91** for the detection of Ba^{2+} ion in aqueous medium facilitated by the use of Triton X surfactant. In the absence of Ba^{2+} **91** displayed characteristic pyrene monomer emission which was found to enhance significantly when Ba^{2+} was added to the solution again due to cancellation of PET. The complexation ability of **91** with Ba^{2+} was enhanced due to the hydrophobic environment of the Triton X micelle. Nakahara *et al.* also developed pyrene functionalized monoaza-18-crown-6 ether derivatives **92a** and **92b,** which demonstrated only a small fluorescent enhancement at relatively high Ba^{2+} concentrations even in the presence of Triton X micelles.[115b] These results suggest that the monoazacryptand scaffold binds Ba^{2+} more strongly than the crown ether moiety even in aqueous micellar environments.

Milton *et al.* prepared PET sensor **93** in a $receptor_1$-$spacer_1$-fluorophore-$spacer_2$-$receptor_2$ format.[116] **93** possessed pyridine and morpholine receptors with a benzimidazolium fluorophore. Addition of Ba^{2+} to aqueous solution containing **93** led to an enhancement of fluorescence, with smaller enhancements also observed for Cr^{3+} and Fe^{3+}. A Job's Plot revealed the binding stoichiometry of **90** with Ba^{2+} was 1:1 host : guest. A ^1H NMR titration of **93** with Ba^{2+} suggested a greater contribution from the morpholine N atom compared to pyridine N atom in binding Ba^{2+}.

5.0 Conclusions

This review examined a range of luminescent probes for the detection of protons, alkali and alkaline earth metal cations. Since the pioneering work of DeSilva in popularizing the PET mechanism almost three decades ago, a multitude of sensors have been developed that operate according to this principle. In the context of designing probes for the ions discussed in this review, the PET principle shines brightly as the tertiary amine electron donor can serve as a building block for the assembly of numerous receptors with defined chelating properties. While not inherently ratiometric, PET probes can easily be adapted to contain a second fluorophore, either as a FRET donor / acceptor or simply to serve as a calibration reference and enable ratiometry. While a lot has been achieved in the past thirty years, new challenges remain. The ready availability of multi-photon excited fluorescence imaging means developing probes with high two-photon absorption cross sections are neccesary for deep tissue imaging applications. In this context, nanoparticle based fluorophores such as Quantum Dots may hold much promise.

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