Part I
Advanced Methodologies

Discovering the Future of Molecular Sciences, First Edition. Edited by Bruno Pignataro.

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Supramolecular Receptors for the Recognition of Bioanalytes

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Detection, identification, and imaging of specific analytes are of broad interest in chemical as well as in biological science. In this regard, molecular sensors play innumerable roles such as in the detection of biological molecules, hazardous materials, and warfare agents, in high-throughput screenings, monitoring biochemical processes, intelligent drug delivery, and molecular logic devices. This chapter focuses on fluorogenic and chromogenic supramolecular sensors for the recognition of important bioanalytes and their applications in various biological studies. A significant amount of literature is available related to this research area [1]. However, our aim is to review the research work carried out by us and selected important examples by others.

1.1
Introduction

Molecular recognition is a basic phenomenon in biological processes. The principle of molecular recognition is the specific interaction between a chemical entity and a target molecule. They are often complementary in their geometric and electronic features [2]. The idea of molecular recognition was first described by Emil Fischer in 1894, who proposed that enzyme and substrate fit together like ''lock-and-key'' [3]. The recognition mechanism is mediated mainly by supramolecular interactions such as hydrogen bonding, ion-pairing, hydrophobic interactions, and dipolar associations [4]. Several examples for these mechanisms exist in nature, for example, deoxyribonucleic acid (DNA) protein, ribonucleic acid (RNA) ribosome, and antigen antibody recognition. Researchers have shown great interest in the design of artificial systems to mimic these biological recognition processes. In this regard, the concept of supramolecular chemistry provides a route to design such sensor materials according to the technical needs [2]. In fact, supramolecular methods have already been proven to be very successful for biomolecule detection. However, developing new methods capable of detecting trace amounts of biologically relevant analytes, such as anions, nucleic acid, enzymes, microorganisms, and proteins in water, is still a demanding task. Apart from detecting methods, the biggest obstacle

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is identifying suitable receptor systems that are sensitive to specific analytes or families of analytes under physiological conditions.

Great advances have been made in the signaling of small target molecules, such as inorganic anions and metal ions [5, 6]. However, it is still difficult to design highly selective and sensitive receptors for complex bioanalytes, such as nucleoside polyphosphates, proteins, nucleic acids, and complex carbohydrates in water. A large number of active research groups around the world, including those of A. D. Hamilton, A. Das, A. Schiller, B. D. Smith, B. Koenig, B. Singaram, C. M. Niemeyer, C. Schmuck, E. V. Anslyn, I. Hamachi, J. L. Sessler, J. -L. Reymond, J. Yoon, J. W. Steed, K. Severin, P. A. Gale, P. Jr. Anzenbacher, R. Jelinek, S. Matile, S. Shinkai, T. D. James, T. Schrader, W. Nau, and many more contributed toward the development of novel supramolecular receptors for the recognition of important bioanalytes.

Fluorescent and colorimetric receptors for binding to bioanalytes are of enormous importance [7]. Fluorescent sensors are crucial as they generally allow detection of the analyte present in (ultra)trace amounts and offer possibilities for the use as a biological cell imaging reagent. In contrast, chromogenic sensors with visual detection have an edge over others as they allow naked eye detection without the use of any sophisticated instrumentation.

1.2 Bioanalytes

It is essential to know the important functions of the target analytes, so that one can design a suitable receptor for them. Our interest and main focus of this chapter lies in pyrophosphate (PPi), nucleoside triphosphates (NTPs), phosphorylated proteins, and peptides, nucleic acids (DNA and RNA), lipopolysaccharides (LPSs), and carbohydrates. These analytes are ubiquitous in nature; phosphates and its derivatives dominate the living world. Most of the coenzymes are esters of phosphoric or pyrophosphoric acid; the principal reservoirs of biochemical energy are phosphates. Many intermediary metabolites are phosphate esters.

PPi ($P_2O_7^{4-}$ (Figure 1.1) is an essential intermediate in biochemical syntheses and degradation reactions [8]. PPi is one of the important products of adenosine-5′-triphosphate (ATP) hydrolysis under cellular conditions, and the detection of PPi has been investigated as a real-time DNA sequencing method [9]. Recently, signaling of PPi has become an important issue in cancer research. Patients with calcium pyrophosphate dihydrate disease (CPPD) have also been shown to have a high synovial fluid PPi level [10].

NTPs (Figure 1.1), such as ATP, cytidine triphosphate (CTP), uridine triphosphate (UTP), are widespread in living systems and crucial for various cellular functions [11]. Among all NTPs, recognition studies of ATP are well known. ATP is produced mainly in mitochondria and used as an universal energy source for various cellular events. It is also involved in enzymatic processes as a reactive substrate. For example, ATP serves as a phosphate donor in kinase catalyzed protein phosphorylation and also acts as an extracellular signaling mediator [12]. Adenosine-5′-diphosphate

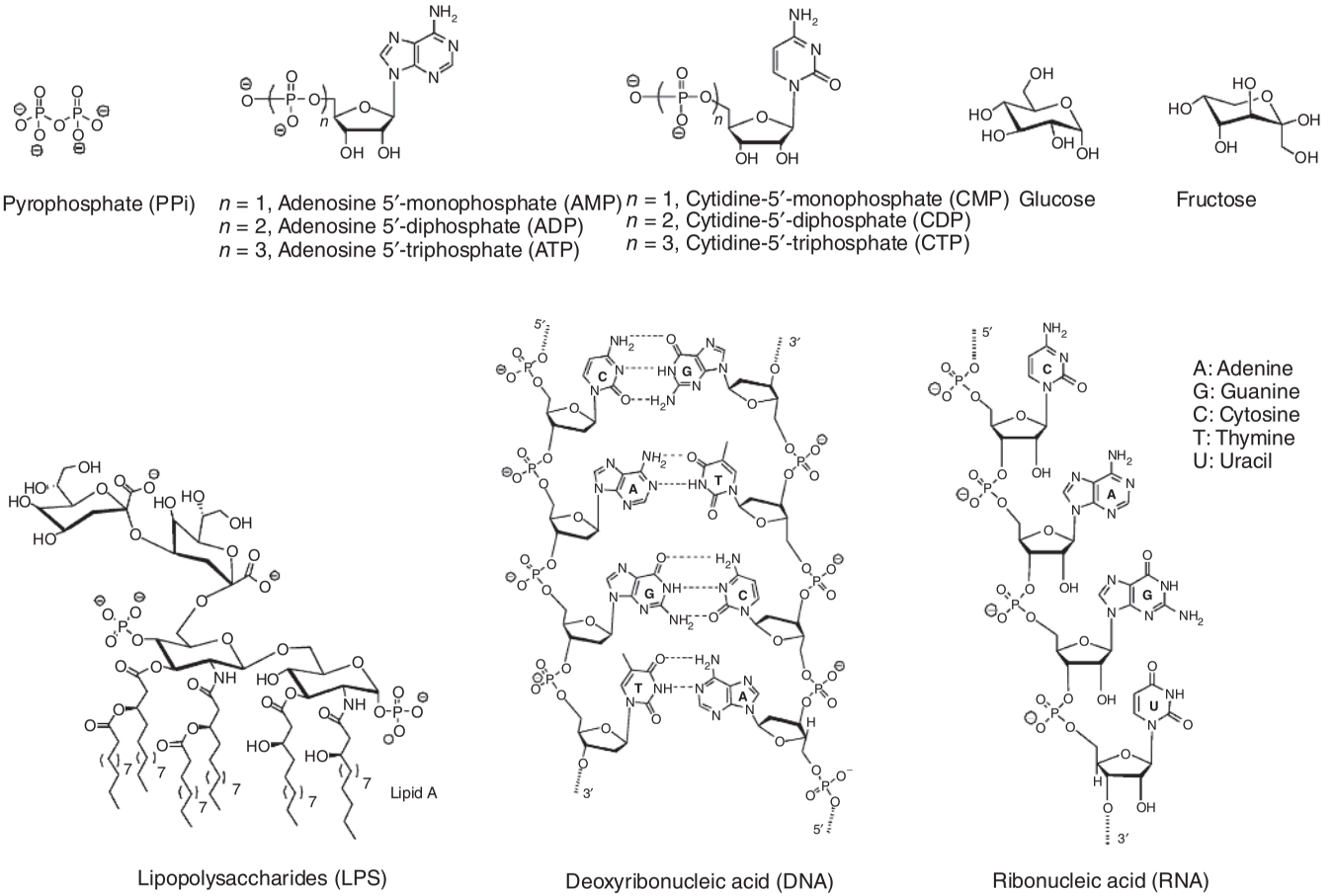


Figure 1.1 Chemical structures of important bioanalytes such as pyrophosphate, nucleoside phosphates (AMP, ADP, ATP, CMP, CDP, and CTP), carbohydrates (fructose and glucose), lipopolysaccharides (LPSs), and nucleic acids (DNA and RNA).

(ADP) and adenosine 5′-monophosphate (AMP) are important for their roles in bioenergetics, metabolism, and the transfer of genetic information.

The genetic materials DNA and RNA are phosphodiesters and they are essential for all known forms of life [11]. DNA is the molecular store of genetic information. The key biological role of RNA is as a messenger; it reads out the genetic code in DNA (transcription) and transports it to the ribosome, where it is decoded into the sequence of a protein (translation) [13]. Single stranded DNAs or RNAs can interact with their complementary strands with high specificity and are useful for nucleic acid detection. Sensor systems for binding nucleic acids have various applications in DNA diagnostics, gene analysis, biological warfare agent detection, forensic investigations, identification of microorganisms in food and environmental samples, and identification of infectious organisms in humans. Currently, the demand for the detection of RNA and DNA sequences for identifying the genetic cause of diseases is rising in medicine [14, 15].

Carbohydrates (saccharides) are known to mediate a large number of biological and pathological events [16]. They are involved in many key biological functions. In the form of glycoproteins, they can alter protein structure and function. As major components of glycolipids, they can play pivotal roles in cell–cell recognition and signaling [17]. They donate nature with structural rigidity, in the form of cellulose, and in the forms of starch and glycogen, they function as the energy store. The simplest biologically important carbohydrates are monosaccharides such as glucose, galactose, and fructose. From a medicinal point of view, the monitoring of glucose has proved of particular importance [18, 19]. In humans, a breakdown in the transport pathways of glucose has been linked to conditions such as cancer and cystic fibrosis [20].

LPSs (Figure 1.1) are amphiphilic molecules present on the outer leaflet of *Gram –ve* bacteria [21]. Despite a great compositional variation depending on their particular bacterial origin, they all consist of a hydrophobic domain known as lipid A (or endotoxin), a nonrepeating ''core'' oligosaccharide, and a distal polysaccharide. LPSs are one of the most potent microbial inducers of inflammation and of a cascade of physiological events that may lead to toxic shock and death. Sensors that are capable of detecting and identifying different types of LPS can be used to develop devices for bacterial diagnostics [22, 23].

1.3 Metal Complexes as Receptors for Biological Phosphates

Molecular recognition for the application in biology must occur at physiological conditions. Thus, receptors should be able to detect phosphates in aqueous or aqueous buffer solution. Mostly, two types of water soluble receptors are found in the literature: (i) positively charged or neutral nonmetallic receptors and (ii) metal complex based receptors [24]. The first type interacts through weak binding forces, such as hydrogen bonding and stacking interactions. The second type interacts mainly with the analyte through charge–charge interactions. The analyte

coordinates to the metal center mimicking many metalloenzymes [25]. Our research interest focuses on both types of receptors. A number of metal ions have been used as receptors for the recognition of biological phosphates (e.g., PPi, NTP), including those of the main group, transition metals, and lanthanides [26]. Researchers have adopted various approaches in the metal-anion coordination to compete with the high hydration energy of phosphates at physiological conditions. However, Zn^{2+} is among the most commonly employed metal center [27]. In addition, coordinatively unsaturated metal complexes as a receptor provide binding sites with high affinity to Lewis bases. Many important bioanalytes (anions, thioles, nucleobases, esters and amides, ureas, etc.) are Lewis bases. They retain a significant affinity even in protic solvents including water.

1.3.1
Fluorescent Zn(II) Based Metal Complexes and Their Applications in Live Cell Imaging

The dipicolylamine (DPA) ligand is often used in zinc complex based sensor systems [28]. It provides a tridentate coordination environment with three nitrogen donors, shows good selectivity for Zn^{2+}, and leaves coordination sites free for anion binding. Two Zn(II)–DPA moieties in a compound exhibit strong binding with biological phosphates in water [28]. Fluorescent chemical receptors based on Zn(II)–DPA complexes for biological phosphates were pioneered by Hamachi and coworkers [29]. They have reported many Zn(II) complexes based on the traditional receptor–linker–fluorophore concept with one or two Zn(II)–DPA moieties as a binding unit, the fluorophore as a signaling unit, and a linker moiety [30].

Xanthene type chemosensor **1** (Figure 1.2a), reported by Hamachi *et al.* [31], was highly selective to nucleoside polyphosphate detection in water. Binding of **1** to ATP leads to a fluorescence turn-on with significant enhancement in the emission intensity (>30-fold). This is actually the recovery of the fluorescence intensity for

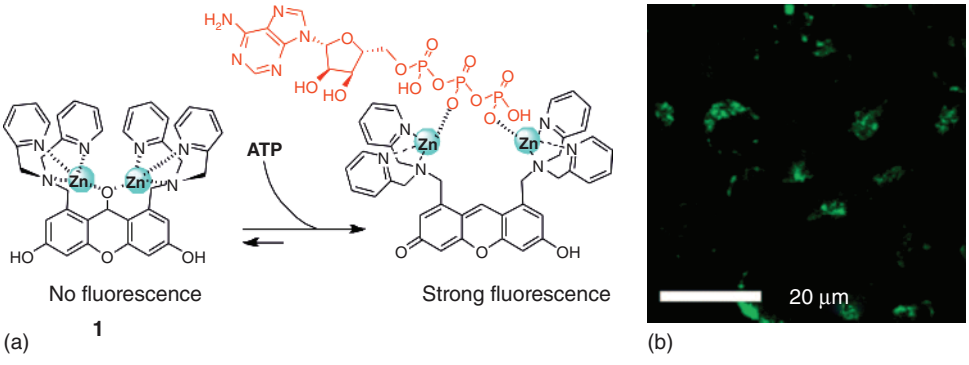
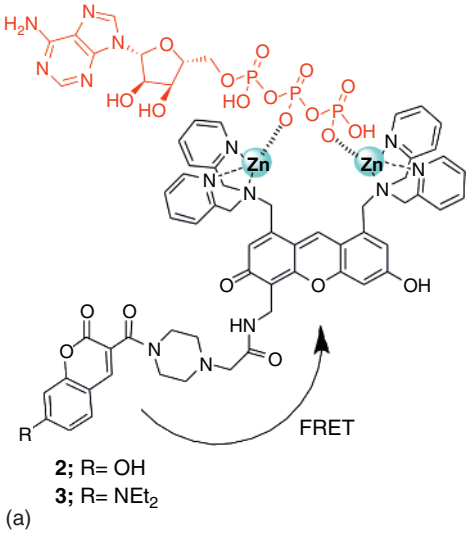


Figure 1.2 (a) Schematic representation of the turn-on fluorescence sensing mechanism of **1** after binding with ATP. (b) Confocal fluorescence images show fluorescence staining of the intracellular ATP stores in Jurkat obtained by **1**. (Adapted and reproduced with permission from Ref. [31]. Reproduced with permission of American Chemical Society (United States).)

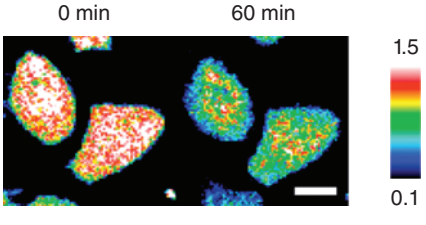
the conjugated xanthene structure, which was quenched after coordination with Zn(II). The association constant for ATP derived from fluorescence titration is $1.3 \times 10^6\ M^{-1}$. The application of **1** as a bioanalytical molecular tool was demonstrated by fluorescence imaging of stored ATP in living Jurkat cells (a human T-cell lymphoblast-like cell line) (Figure 1.2b).

The group of Hamachi has also developed fluorescence resonance energy transfer (FRET) based ratiometric sensors **2** and **3** (Figure 1.3a) for the detection of ATP [32]. It was shown that, for receptors **2** and **3**, the same xanthene backbone of **1** acts as FRET acceptor along with a coumarin FRET donor. After binding with ATP, FRET from the coumarin to the xanthenes was observed. The affinity constant of these receptors toward ATP was calculated to 2.9×10^6 and $7.3 \times 10^6\ M^{-1}$, respectively, in aqueous solution. However, no detectable emission change was observed with monophosphates and phosphodiester species. The significant ratiometric sensing of ATP was used for the real-time visualization of the ATP level inside HeLa cells (cell lines HEK293 and NIH3T3) and the monitoring of two enzyme reactions involving nucleoside polyphosphates. Though these two chemosensors show relatively low selectivity among the polyphosphates, these are applicable to image the ATP level, inside the living cells (Figure 1.3b).

Very recently, the same group also achieved the imaging of nucleoside polyphosphates on plasma membrane surfaces with a lipid modified receptor **4**, which has a same xanthene core as receptor **1** (Figure 1.4). Receptor **4** was able to detect

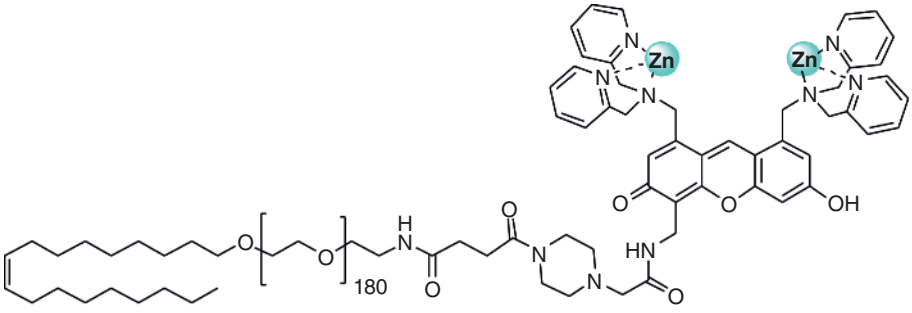


(a)



(b)

Figure 1.3 (a) Molecular structures for **2** and **3** and the FRET via turn-on fluorescence sensing on binding with ATP. (b) Ratiometric analysis of living cells stained with **2**. Pixel-by-pixel ratio image of a HeLa cell before (0 min) and after (60 min) treatment with 20 mM 2-deoxyglucose and 1 mM potassium cyanide (KCN). Scale bar: 10 µm. (Adapted and reproduced with permission from Ref. [32]. Reproduced with permission of American Chemical Society (United States).)



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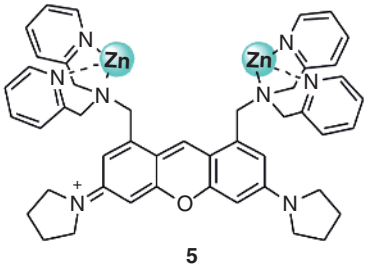

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Figure 1.4 Receptors used for site-specific imaging of nucleoside polyphosphates.

polyphosphate derivatives XTP (X = A, G, C), XDP (X = A, U), and PPi with a several-fold enhancement in fluorescence intensity.

In a live cell imaging study, sensor **4** with a lipid anchor selectively localized on the plasma membrane surface and detected the extracellular release of nitrophenyl-phosphates (NPPs) during cell necrosis induced by streptolysin. For subcellular imaging of ATP in mitochondria, they have also designed rhodamine-type Zn(II) complex **5**, possessing a cationic pyronin ring instead of xanthenes. Receptor **5** detects the local increase of ATP concentration during apoptosis. Multicolor images were obtained through simultaneous use of **4** and **5** allowing detection of the dynamics of ATP in different cellular compartments at the same time [33].

1.3.2
Chromogenic Zn(II)-Based Metal Receptors and Their Applications in Biological Cell Staining

Most of the existing reports on the recognition of biological phosphates are based on changes in fluorescence properties [34]. Examples for colorimetric detection of biological phosphates in aqueous environments are rare in literature [35]. In an effort to make colorimetric receptors for the recognition of biological phosphates, Amilan Jose *et al*. reported a new chromogenic complex **6**, which can be used to bind ATP in aqueous solutions under biological conditions [36, 37] (Figure 1.5).

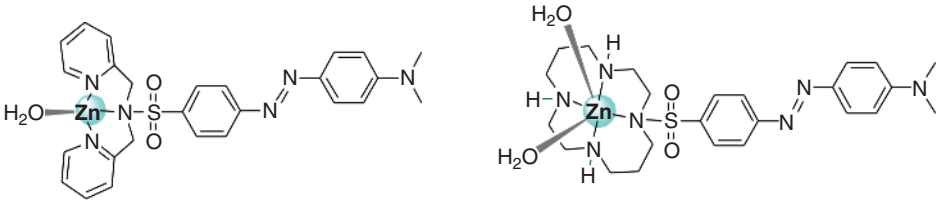

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Figure 1.5 Chemical structures for receptors **6** and **7**.

A Zn(II)–DPA unit in receptor **6** acts as a receptor fragment for ATP recognition, while the dimethylamino phenylazo group acts as the signaling unit for reporting the binding, detectable by a color change.

The selectivity of the receptor **6** toward different biologically important anions was checked in aqueous media. The absorption maximum of **6** was found to be shifted to 484 nm from 463 nm with ATP (Figure 1.6a). However, on addition of ADP, a much smaller red shift (8 nm) in λ_{max} occurred and no distinct change in color could be

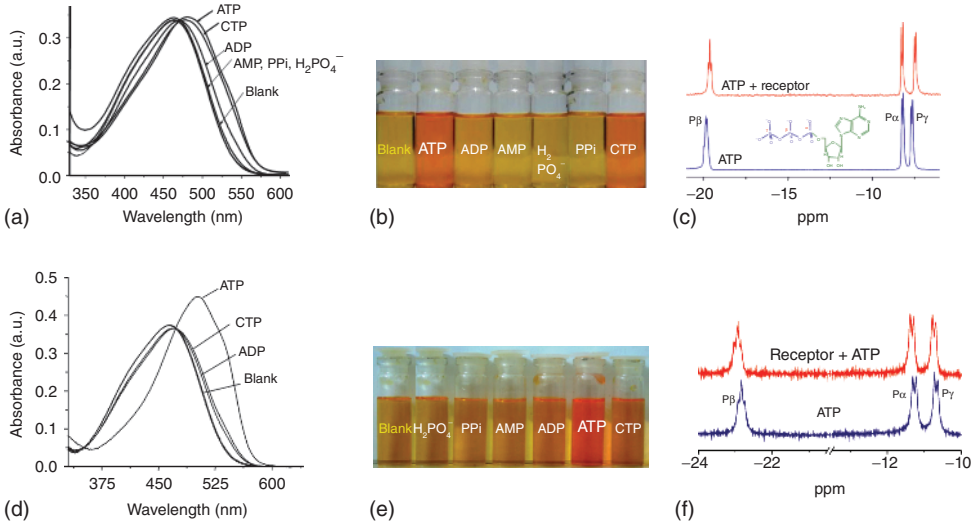


Figure 1.6 (a) Absorbance spectra of **6** (25 μM) in HEPES buffer solution (pH 7.2) at 25 °C in the presence of various anions (250 μM) (b) change in color of **6** in aqueous solution; from left to right: blank, with ATP, ADP, AMP, $H_2PO_4^-$, PPi (anion concentration 100 μM), and CTP (125 μM). (c) Partial ^{31}P-NMR spectrum of ATP in the presence and absence of receptor **6**. (Adapted and reproduced with permission from Ref. [36]. Reproduced with permission of American Chemical Society (United States).) (d) Absorbance spectra of **7** in HEPES buffer solution (pH 7.2) at 25 °C in the presence of various anions (e) change in color of **7** in aqueous solution with ATP, ADP, AMP, $H_2PO_4^-$, PPi, and CTP. (f) Partial ^{31}P-NMR spectrum of ATP in the presence and absence of receptor **7**. (Adapted and reproduced with permission from Ref. [38]. Reproduced with permission of Royal Society of Chemistry (United Kingdom).)

seen by the naked eye (Figure 1.6b). Furthermore, no change in absorption spectra was observed on addition of AMP, PPi, or $H_2PO_4^-$ (Figure 1.6a). For experiments with excess CTP, an almost similar spectral shift was detected as for ATP. Binding constants for ATP and CTP, evaluated from spectrophotometric titration, were found to be 1130 ± 6 and $772 \pm 5\ M^{-1}$, respectively, in aqueous solution (pH ~ 7.2) at 25 °C. The change in color and spectral behavior on binding of ATP to the Zn(II) center in **6** was associated with the perturbation of the energy of the frontier orbitals of the donor amine functionality and the acceptor azo fragment [36]. Binding of ATP was also confirmed by ^{31}P-NMR spectroscopy (Figure 1.6c). Downfield shifts for the ^{31}P signals for γ and β-P atoms signify the binding to Zn-atom of **6** through the oxide of respective phosphate units. An insignificant shift in ^{31}P signals was observed when similar experiments were repeated for ADP, and no shift was observed with AMP. The enhanced electrostatic interaction between triphosphates and **6** is crucial for efficient **6**-O (phosphate) binding compared to other anions. The observed binding preference for ATP or CTP ≫ ADP ≫ AMP could be attributed to the difference in the number of the anionic charges of the phosphate species [36].

Saccharomyces cerevisiae (yeast cells) is a eukaryotic microbe that derives its energy in the form of ATP. The surface of these yeast cells is exposed with negatively charged ATP. The chemosensor **6** could be used for the staining of this type of cells. The colorless yeast cells were viewed under normal light microscopy (Figure 1.7) with and without exposition of **6**. The microscopy images revealed that the treated cells got stained with **6** and the color of the cells changed to reddish yellow (Figure 1.7a).

The change in the color of yeast cells indicates that the negatively charged phosphate groups on the surface of the cells were effectively bound to **6**. The

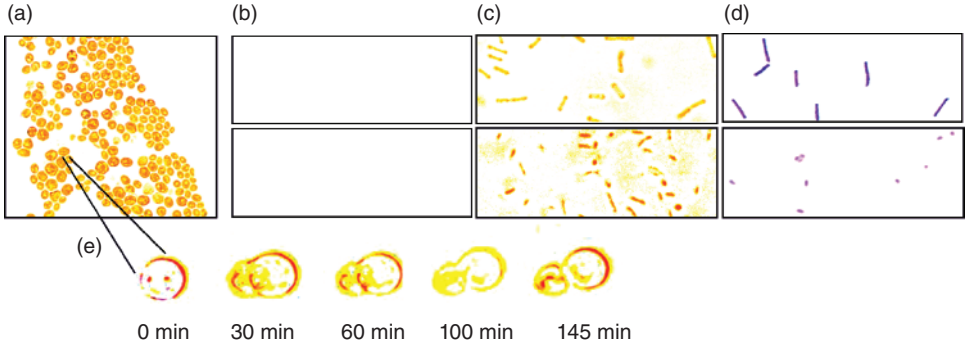


Figure 1.7 Light microscopy images (a) yeast cell with receptor **6**, (b-top) *Gram +ve* Bacillus sp. without any staining agent, (c-top) when treated with **6**, (d-top) when treated with gentian violet dye; *Gram −ve* Pseudomonas sp. (b-bottom) without any staining agents, (c-bottom) when treated with **6**, and (d-bottom) when treated with safranin dye. (e) Light microscopy images of a yeast cell stained with **6** monitored at different time intervals. (Adapted and reproduced with permission from Ref. [36]. Reproduced with permission of American Chemical Society (United States).)

staining was found to be stable as the color of the stained cells remained unchanged even after subsequent washing with water/ethanol. The possibility of using **6** as staining agent for prokaryotes (*Gram +ve* and *Gram −ve* bacterial cells) was also investigated. The experimental results suggested that the single staining agent **6** could distinguish between *Gram +ve* and *Gram −ve* bacteria through distinctly different color intensities and shape of the stained cells. The difference in the staining intensity for two different bacteria can be better understood if one considers the difference in cell structure and cell wall composition of the respective bacteria.

The viability of the cells was observed before and after staining with **6** under the light microscope. Unaffected cell proliferation and growth confirmed that the staining agent **6** was nontoxic and kept cells viable after staining (Figure 1.7e). The growth dynamics was monitored for the same eukaryotic cells and prokaryotic bacteria in an aqueous culture medium in the absence and presence of **6** by recording the change in absorbance spectrum as a function of time. The plot of change in absorbance versus time shows a nice correlation between the cell growth and the ATP generation with progressive growth (lag phase to log phase and then stationary phase and ultimately a decline in the growth curve) for the respective cells during the metabolic processes. The growth profile was also monitored in the absence and presence of two different respiratory inhibitors such as rotenone and cycloheximide (which reduces the ATP generation and cell growth, respectively). Studies with respiratory inhibitors confirm the staining due to the binding of extracellular ATP, produced *in situ*, with **6**.

In a subsequent work by the same group, Zn(II)–cyclam modified chromogenic complex, **7** and its [2]-pseudorotaxane form, **α-CD**.**7** (CD = cyclodextrin) was reported for preferential binding of ATP, among other biologically important phosphates in aqueous solution (Figures 1.6 and 1.8) [38, 39]. The chemosensor **7** exhibits higher solubility in aqueous medium as well as improved selectivity toward ATP. A visually detectable change in solution color was observed on addition of ATP to **7** (Figure 1.6e). The charge transfer absorption band of **7** at 463 nm was red shifted with a maximum at 503 nm on addition of excess sodium salt of ATP. An insignificant shift of 9 nm was observed when CTP of comparable concentration was added (Figure 1.6d). In contrast, there was almost no change in the spectra and color with other phosphate anions. This confirms that receptor **7** is very selective for ATP among other nucleoside phosphates examined. The relative binding affinity for ATP of $K = 1.9 \times 10^3\ M^{-1}$ was evaluated by systematic titrations in *N′*-2-hydroxyethylpiperazine-*N′*-2 ethanesulfonic acid (HEPES) buffer medium.

The binding affinity of the ATP with **7** is slightly higher than that of **6**. Unlike receptor **6**, ^{31}P–NMR binding studies of **7** with ATP shows upfield shifts for the α-, β-, and γ-phosphorus atoms of the bound ATP (Figure 1.6f). These chemical shifts signals signify the binding to Zn-atom of **7** through oxygen atom bearing the negative charge of the respective phosphate unit. Relatively weaker interaction of the $O_{[\gamma-PO4]}^{-}$ and $O_{[\alpha-PO4]}^{-}$ relative to $O_{[\beta-PO4]}^{-}$ unit accounts for the smaller $\Delta\delta$ shift in ^{31}P–NMR spectra. This observation validates the formation of a hepta coordinated Zn(II)-center in **7** in presence of ATP. The solubility of the **7** (0.045 g l^{-1}) in water was found to be enhanced (0.34 g l^{-1}) in the presence of α-CD. This is

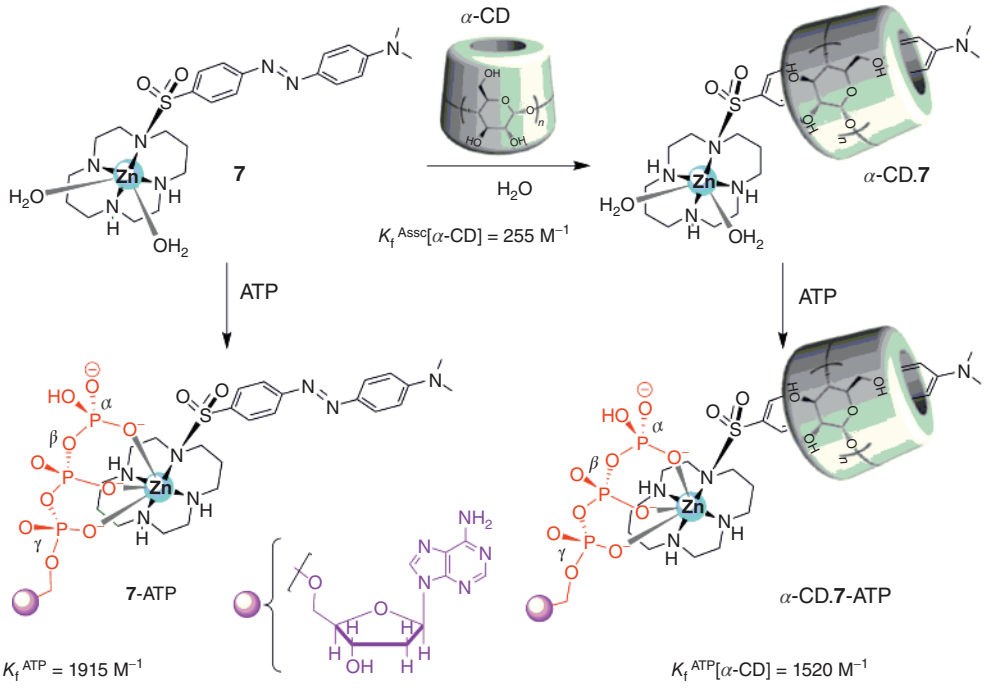


Figure 1.8 Schematic representation of the formation of [2] pseudorotaxane, **α-CD-7**, and the binding of ATP to the Zn(II)-center of **7 or α-CD-7**. (Adapted and reproduced with permission from Ref. [38]. Reproduced with permission of Royal Society of Chemistry (United Kingdom).)

due to the favored nonbonded interactions after inclusion of the hydrophobic azo functionality of **7** into the hydrophobic cavity of the α-CD. High solubility of **7** in water in the presence of α-CD helped to attain a higher effective concentration of **7** and an intensified color change on binding to ATP [38].

The possible use of 7 and **α-CD.7** as staining agents for yeast cells was also studied. The results demonstrated that it could be used as a colorimetric staining agent for eukaryotic yeast cells and can be viewed under a simple light microscope. Staining studies were also conducted with prokaryotic *Bacillus sp.* (*Gram +ve*) and *Pseudomonas sp.* (*Gram −ve*) bacteria (Figure 1.9). The *Gram +ve* bacteria appeared longer in the images as expected, while more intense staining was observed for *Gram −ve* bacteria. After staining, the color of the *Gram +ve* bacteria cells changed from colorless to pink, but in the case of *Gram −ve* bacteria the color change occurred from colorless to violet. The thinner, hydrophilic, and more porous cell walls of the *Gram −ve* bacteria are expected to allow higher excretion of ATP to the cell surface, where it gets bound to the **6**, **7**, and **α-CD.7** thereby causing the efficient staining.

Similarly to **6**, the experimental results again confirmed the retained viability of all the live cells after staining with both **7** and **α-CD.7** and this nontoxic behavior

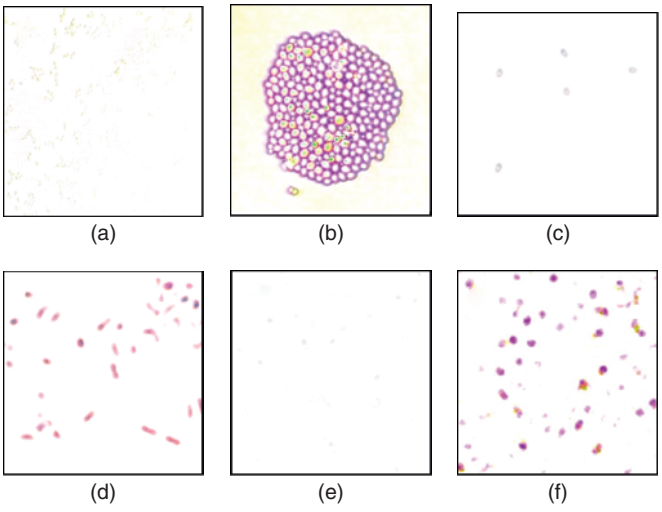


Figure 1.9 Light microscopic images of cells with **α-CD.7** (a) unstained *S. cerevisiae* cells, (b) stained *S. cerevisiae* cells, (c) unstained *Gram +ve* bacteria, (d) stained *Gram +ve* bacteria, (e) unstained *Gram −ve* bacteria, and (f) stained *Gram −ve* bacteria at 25 °C in 10 mM HEPES buffer solution. (Adapted and reproduced with permission from Ref. [38]. Reproduced with permission of Royal Society of Chemistry (United Kingdom).)

could be used for studying the cell growth dynamics of each of these individual microbes [40]. Thereby, colorimetric receptors **6**, **7**, and **α-CD.7** can be useful as efficient viable staining agents for a microorganism through selective recognition of biological phosphate anion. In addition, other interesting Zn(II) complex based receptors for the recognition of PPi and simple phosphate have also appeared in literature [26, 41–44].

1.4 Functionalized Vesicles for the Recognition of Bioanalytes

Vesicular particles are an interesting class of dynamic supramolecular structures and have been employed in diverse applications in biological research, mostly because of their relative ease of preparation and variability in composition [45–47]. Vesicles are often perceived as closely mimicking the cell membrane [48, 49]. These features have promoted the use of vesicles in molecular recognition [50]. Our research interest focused on the development of new color and fluorescence based vesicles for the recognition of bioanalytes [51, 52]. In particular, we were interested in polydiacetylene (PDA) based vesicles as receptors for the detection and analysis of biological analytes. In this section, we describe our research and the ensuing results of PDA vesicles for biomolecular sensing and very interesting examples of other research groups.

1.4.1 Polydiacetylene Based Chromatic Vesicles

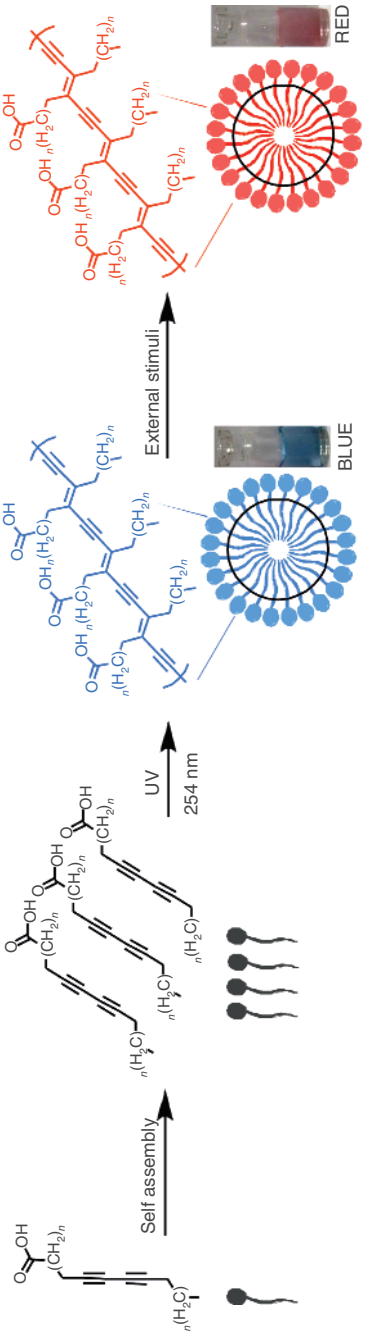
Conjugated PDAs are an amazing polymeric system that displays unique chromatic properties [53, 54]. PDA polymers are formed by the 1,4-addition of self-assembled diacetylenic monomers; the reaction is initiated by ultraviolet (UV) irradiation at 254 nm (Scheme 1.1). The resulting polymer is intensely blue in color. Electronic delocalization within the conjugated framework results in an absorption at around 650 nm [55]. The practical use of PDAs arises from their ability to undergo a blue to red visible color transitions in response to different external stimuli, such as temperature, pH, mechanical stress, and chemical and biological species.

The mechanism corresponding to the color change is believed to be an irreversible stress-induced structural transition of the conjugated backbone of the polymer. This direct colorimetric detection strategy bypasses the need for optical reporters and transducers [56, 57]. The lipido-mimetic nature of PDA, that is a hydrophobic tail (long aliphatic chain) and a hydrophilic headgroup (carboxylate), results in the formation of biomimetic assemblies, such as nanoscale vesicular particles in aqueous solutions and monolayers at the air/water interface.

This unique behavior of stimuli-induced blue to red color transition as well as fluorescence enhancement of the PDAs has led to the development of a variety of PDA-based sensing components. Mostly, PDA sensors have been used as thin films or as vesicles in solution. A bottleneck in the development of a PDA sensor assembly for molecular recognition is the preparation of the diacetylene monomers embedded with the suitable recognizing element of choice. Two important approaches have been used to functionalize the surface of the assembly. In the first case, the diacetylenic monomer lipid is covalently modified with the appropriate receptor by synthetic reaction. This allows direct cross-linking of the ''receptor-lipid'' with the surrounding PDA matrix. In the second case, a receptor molecule is noncovalently incorporated into the PDA matrix analogous to the heterogeneous mixing of molecules in cell membranes [58, 59]. One of the initial demonstrations of PDA sensor for the potential biological application is the colorimetric detection of influenza virus by the sialic acid ligand modified PDA films [60].

1.4.1.1 PDA Based Receptors for Biological Phosphate

Metal ion functionalized vesicles also play an important role for molecular recognition at membrane–water interfaces [49, 61]. The ability to modify the vesicle membranes with metal complex based receptors for biological analytes is an important aspect and immature area in molecular recognition. As described in the previous section, metal complexes, such as Zn(II)–DPA and Zn(II)–cyclen (cyclen = 1,4,7,10-tetraazacyclododecane), can reversibly coordinate anionic analytes of biological origin under physiological conditions with high affinity and selectivity. Many research groups have investigated the application of Zn(II)–DPA receptors in liposomes for binding with biological analytes, molecules transport across membranes, and cell staining [30, 45, 47, 61–63].



Scheme 1.1 Structural and chromatic properties of polydiacetylene (PDA) based vesicles as receptors.

However, the preparation of self-assembled PDA vesicles with biological phosphate binding amphiphilic Zn(II)–cyclen and Cu(II)–IDA (IDA = iminodiacetato) complexes (Figure 1.10) was reported by Amilan Jose *et al*. for the first time [52]. The receptor functionalized liposomes **LP-8**, **LP-9**, and **LP-10** were prepared by a proper mixture of mono, bis-Zn(II)–cyclen, and Cu(II)–IDA receptor modified diacetylene monomer and the unmodified diacetylene monomer 10,12-tricosadiynoic acid (TCDA) or 10,12-pentacosadiynoic acid (PCDA) in buffered aqueous solution (10 mM, HEPES, pH 7.2). The polymerized self-assembled bilayer vesicles were prepared at room temperature by irradiating the solutions with light at 254 nm, whereby the colorless receptor embedded vesicle solution turned blue. The average size of the liposomes of 160–200 nm was determined by dynamic light scattering (DLS). The absorption spectra of the modified vesicles show distinct absorption bands at 640, 589, and 543 nm. However, on addition of ATP and PPi to the **LP-8** and **LP-9** PDA vesicles, the absorption band at 640 nm disappeared completely and intense absorption bands at 489 and 543 nm were observed (Figure 1.11a). The color of the solution turned red. No changes in the absorption spectra or color were

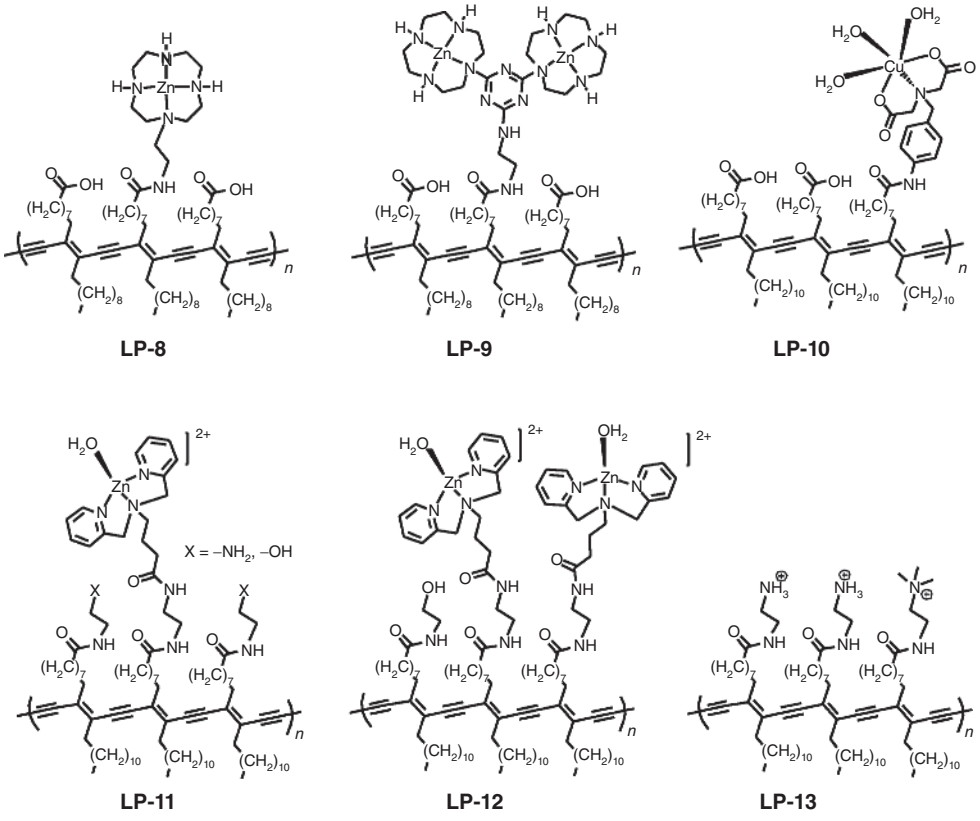


Figure 1.10 Polymeric PDA vesicles prepared from receptor modified diacetylene monomers.

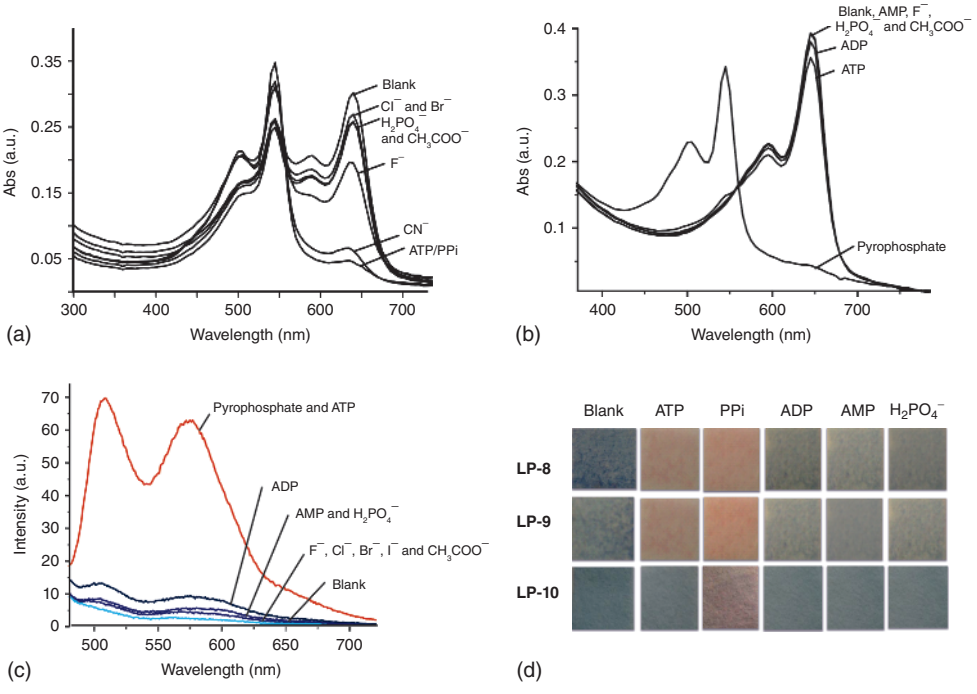


Figure 1.11 (a) UV-visible spectra of **LP-9** in the presence of different anions (aqueous solution, HEPES 10 mM, pH 7.2, 100 equiv. of the anion salt added). (b) UV-visible spectra of **LP-10** in the presence of different anions (aqueous solution, HEPES 10 mM, pH 7.2, 100 equiv. of the anion salt added). (c) Emission spectra of **LP-9** in the presence of different anions (aqueous solution, HEPES 10 mM, pH 7.2, 100 equiv. of the anion salt added). (d) Color change of the receptor embedded test paper with different anions. (Adapted and reproduced with permission from Ref. [52]. Reproduced with permission of Wiley-VCH (Germany).)

observed with other anions such as F^-, Cl^-, Br^-, I^-, $H_2PO_4^-$, CH_3COO^-, AMP, or ADPunder similar conditions.

The color change of the Zn(II)–cyclen modified PDA liposomes from blue to red was quantified via the colorimetric response (CR) by using equation $\%CR = [(A_0 - A_x)/A_x] \times 100$. The absorption ratio before analyte addition is calculated as $A_0 = I_{620}/(I_{620} + I_{490})$ and the absorption ratio after analyte addition followed from $A_x = I_{620}/(I_{620} + I_{490})$, respectively. **LP-9** prepared from dinuclear Zn(II)–cyclen complexes showed an increased affinity to ATP and PPi ions than **LP-8** prepared from mononuclear Zn(II)–cyclen complexes. It was interesting to see that Cu(II)–IDA complex modified vesicles (**LP-10**) behave differently with biological phosphates as compared to Zn(II)–cyclen modified vesicles. Among different phosphates, **LP-10** responded only to PPi and is able to selectively discriminate between ATP and PPi (Figure 1.11b). The binding of ATP and PPi with vesicular receptors were also monitored by emission intensities. The emission spectra of **LP-8**, **LP-9**, and **LP-10** in aqueous buffered solution showed very weak

emission bands centered at 625 nm on excitation at 510 nm. The intensity of the emission band increases significantly in the presence of ATP and PPi. Other anions induce only very little or no change in the emission intensities (Figure 1.11c). The intrinsic response of colorimetric vesicles can provide convenient ''naked eye'' detection and the corresponding analyte affinities are usually in the millimolar (mM) range. For practical applications, test papers of the vesicles were prepared by soaking filter papers in the solution of the vesicles and drying them in air. The blue colored test paper was immersed in the aqueous analyte solution for several seconds and then air-dried. Similarly to solution, the color of the test paper made from **LP-8** and **LP-9** changed with aqueous solutions of ATP and PPi. However, in the case of **LP-10**, only with PPi the color change was obtained (Figure 1.11d).

Ahn *et al*. reported that similar family of PDA vesicles functionalized with Zn(II)-DPA binding unit in solution and solid substrates for PPi [64]. A functionalized liposome (**LP-11**) has been prepared by an 1 : 1 mixture of Zn(II)-DPA functionalized PDA monomer and ethylene diamine (EDA) capped PDA (**PCDA-EDA**) monomer. This solution (0.25 mM HEPES, pH 7.4) was irradiated at 254 nm and treated with zinc nitrate to obtain the corresponding polymerized liposome **LP-11**.

Scanning electron microscopy and DLS analyses confirmed the size distribution range of 40–80 nm. In these cases blue liposome solution (**LP-11**, where $X = NH_2$) became red–purple on interaction with only phosphate or PPi; no color change was observed with the other anions. Even though color changes were observed with ATP and AMP, they caused precipitation, which made them not suitable for further studies. The observed analyte selectivity can be explained by evoking the strong affinity of the Zn(II)-DPA ligands toward phosphate and PPi anions. In contrast, little color change was observed on addition of anions to a solution of **LP-11** (where X = OH) suggesting that the presence of amino group also plays a role in anion recognition. The CR values show the high selectivity of the liposome toward phosphate and PPi ions in solution. PPi selective **LP-11** also successfully fabricated into microarray chip. The microarray-chip system selectively responds to PPi and the red fluorescence spot images are clearly visible in the presence of 1 μm to 1 nm range of PPi (Figures 1.12a–j).

Recently, the same group has prepared **LP-12** by mixing 2 : 1 mixture of Zn(II)-DPA functionalized PDA monomer and alcohol-terminated PDA monomer. **LP-12** and **LP-11** differ from PDA monomer ratio used for the liposome preparation. **LP-12** shows a color change from blue to reddish purple and emits fluorescence in the turn-on mode on interaction with phosphatidylserine over other analytes such as phosphatidylcholine, sphingomyelin, and phosphatidylethanolamine [65]. Confocal fluorescence microscopy and fluorescence-activated cell sorting (FACS) analysis demonstrate that liposome responds to apoptotic cells and selectively stains the apoptotic cells in a manner similarly to commercial apoptosis detection kit (Figure 1.12k–n). Cell staining study also demonstrates that liposome can be used to detect apoptotic cells over normal cells.

Quaternary ammonium and primary amine head groups modified PDA sensor system (**LP-13**) for biological phosphates was reported by Juyoung Yoon *et al*. [66]. The sensor displayed a selective colorimetric change and a large fluorescence

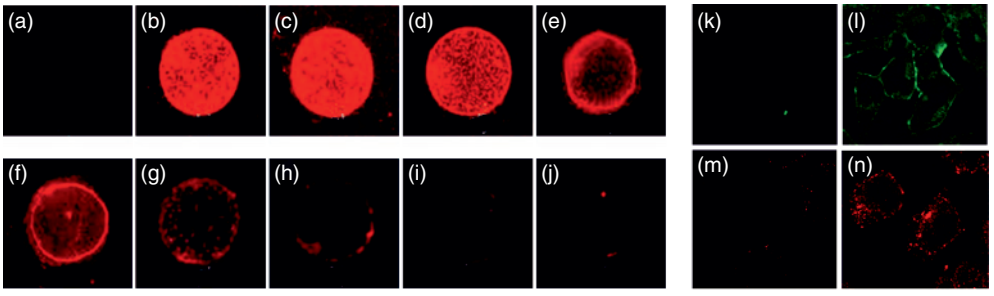


Figure 1.12 Fluorescence images of the liposome chip to pyrophosphate at various concentrations: (a) buffer only, (b) 100 µm, (c) 10 µm, (d) 1 µm, (e) 100 nm, (f) 10 nm, (g) 1 nm, (h) 100 pM, (i) 10 pM, and (j) 1 pM. The images were taken after dipping the chip into each analyte solution (10 mM, pH = 7, HEPES buffer) and incubating for 6 h at room temperature. (Adapted and reproduced with permission from Ref. [64]. Reproduced with permission of Wiley-VCH (Germany).) and confocal fluorescence microscopic images of HeLa cells in HEPES buffer (normal cells: k, l and apoptotic cells: m, n) stained with fluorescein conjugated Annexin V from Aldrich (k,l) and **LP-12** (m,n), respectively. (Adapted and reproduced with permission from Ref. [65]. Reproduced with permission of Wiley-VCH (Germany).)

enhancement in the presence of ATP at pH 7.0 in water among various anions. The ratio between PDA monomers and the control of steric factors were critical points for ATP detection. The possible interaction of phosphate groups in ATP with quaternary ammonium units and ammonium units on the surface of the PDA polymer could be the reason for the selectivity of vesicles toward ATP.

1.4.1.2 PDA Based Receptors for Lipopolysaccharide

LPS is highly toxic and biologically active. Owing to its high toxicity, continuous effort has been directed toward the development of specific detection of LPS. PDA liposomes prepared from amino acids functionalized PDA monomers (**14** and **15**) were used for the detection of LPS from five different strains of *Gram –ve* bacteria such as *Escherichia coli O26 : B6*, *Pseudomonas aeruginosa*, *Salmonella minnesota*, *Shigella flexneri*, and *Salmonella enteriditis* [67]. On interaction of liposome with LPS, the color change of the liposomes from blue to red was quantified by calculating the CR value. Four different conditions (i) at RT, (ii) at 35 °C, (iii) with sodium dodecyl sulfate (SDS), and (iv) with ethylenediamine tetraacetic acid (EDTA) were used to get eight CR values for two PDA liposomes prepared from **14** and **15**. The fingerprints obtained from a set of five LPS are unique enough to identify all five LPS unambiguously in a blind test [67].

The group of Schmuck *et al*. reported a peptide-functionalized PDA liposome as a turn-on fluorescent sensor for LPS at micromolar concentrations in water [68]. Inspired by naturally occurring antibiotic polymyxin B (PMB), they designed new fluorescent sensors for LPS. Two diacetylene monomers connected with histidine (**16**) and pentalysine oligopeptide (**17**) have been synthesized by using microwave-assisted peptide synthesis (Figure 1.13). Irradiating a 1 : 9 mixture of highly fluorescent self-assembled PDA monomer (**16** and **17**) led to a complete

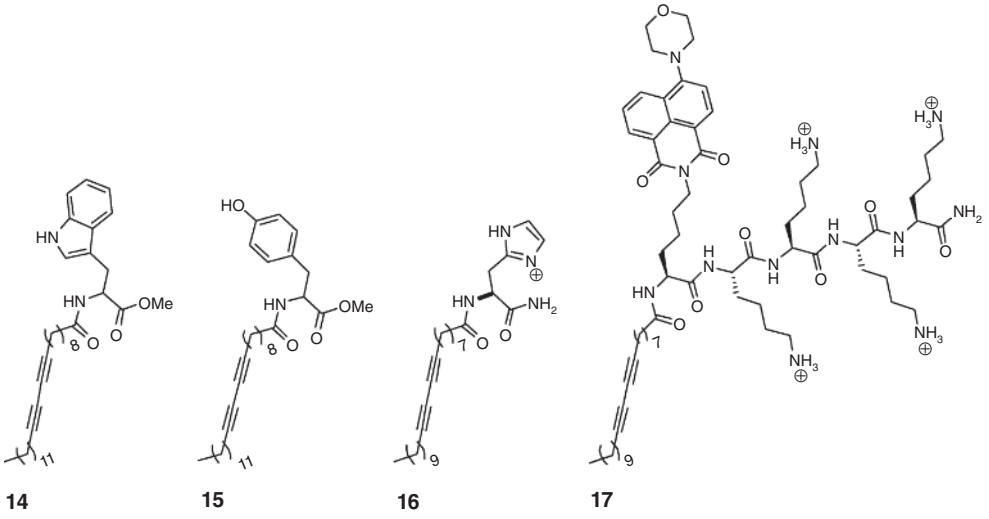


Figure 1.13 Chemical structure of receptor modified PDA monomers for LPS detection.

quenching of the fluorescence. This quenching is caused by an energy transfer from the napthalic acid fluorophore (emission maximum at 540 nm) to the cross-linked polymer (absorption maximum at 536 nm). Binding of submicromolar concentration of LPS to the nonfluorescent polymerized PDA liposomes restored the fluorescence. The fluorescent change is only selective for LPS, compared to other anionic biological relevant species, such as nucleotides, anionic sugars, or ctDNA. Stern-volmer analysis provided a binding constant of $K = 1.5 \times 10^6\ M^{-1}$ (Figure 1.14). Further LPS selective sensor allowed for the fluorescence staining of the membrane of *E. coli* bacteria; control experiment also showed that the LPS selective PDA liposomes are nontoxic to either bacteria or human cells.

1.4.1.3 PDA Based Receptors for Oligonucleotides and Nucleic Acids

Sequence-specific DNA detection is important in medical, biological, and biotechnological areas. Techniques for detection of small quantities of DNA find broad potential applications including gene expression monitoring, pharmacogenomic research, drug discovery, viral, bacterial, forensic, and genetic identification [69].

Ma and coworkers developed a colorimetric method for the detection of oligonucleotides by PDA liposomes [70]. PDA vesicles were prepared by the mixture of **TCDA** (70%), dimyristoylphosphatidylcholine (**DMPC**, 29 mol%), and respective oligonucleotides (Probe 1 or Probe 2, 1%). Oligonucleotides were partially complementary to opposite ends of the target DNA. In the presence of the target DNA, 5′-TACGAGTTGAGAATCCTGAATGCG-3′, the PDA vesicles experience a chromic transformation from deep blue to red (Figure 1.15). The force produced on the conjugated backbones of liposomes leads to color transitions that come from the hybridization of two oligonucleotides with target DNA. On the other hand, on addition of mismatched oligonucleotides, no color or absorption spectrum

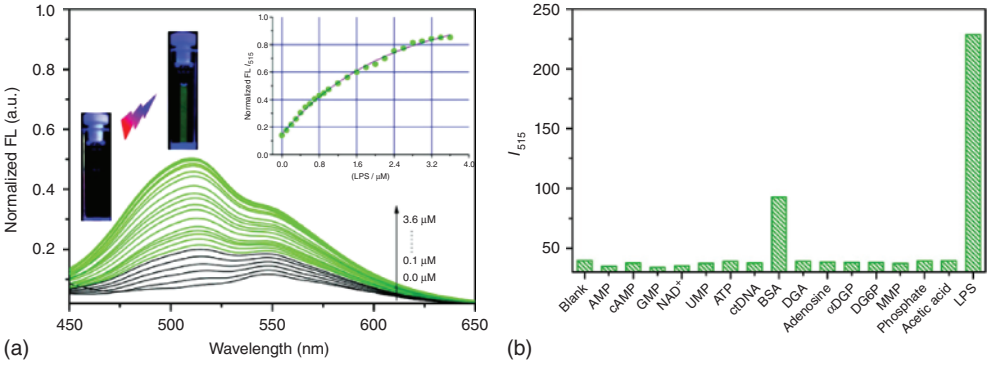


Figure 1.14 (a) Fluorescent emission titration spectra of the PDA liposomes prepared from 10 : 90 mixture of **16** and **17** with LPS in 10.0 mM DMSO/TBS (v/v = 1/4, pH = 7.4). Inset: Normalized fluorescence intensity at 515 nm versus the concentration of LPS (0–3.6 μM). (b) The selectivity of the increase of fluorescence on addition of various biologically important species. Only LPS (and to a much lesser extent the protein BSA) gives rise to a significant increase in fluorescence. (Adapted and reproduced with permission from Ref. [68]. Reproduced with permission of American Chemical Society (United States).)

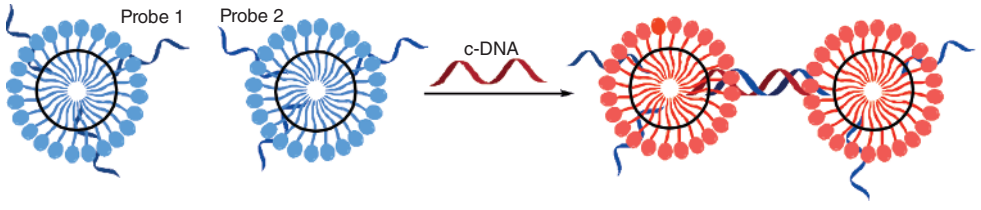
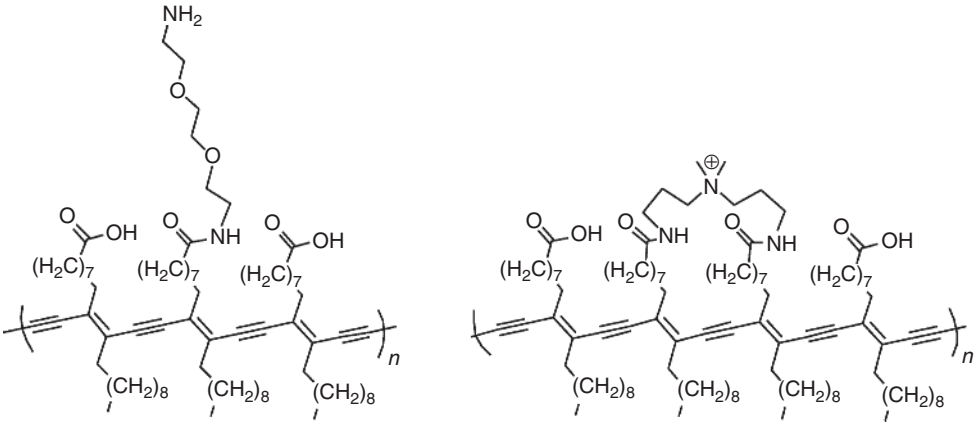


Figure 1.15 Schematic diagrams for the colorimetric detection of DNA using polydiacetylene vesicles functionalized with probe DNA. The sequences of different oligonucleotides used for the study is Probe 1: 5′-TCTCAACTCGTATTTTTT-$(CH_2)_3$-cholesteryl-3′; Probe 2: 5′-cholesteryl-$(CH_2)_3$-TTTTTTCGCATTCAGGAT-3′; target DNA: 5′-TACGAGTTGAGAATCCTGAATGCG-3′; mismatched DNA: 5′-GCGTAACTCCTAAGAGTTGAGCTA-3′. (Figure adapted from Ref. [70].)

changes were observed. These results indicate that the sensing system could be highly specific to target DNA sequences. However, this strategy is tedious and time-consuming because PDA liposomes with different probe oligonucleotides need to be prepared depending on the target DNA.

A novel strategy for the detection of nucleic acids was developed by Kim and Park *et al.* It is based on the positively charged PDA vesicles and negatively charged phosphate backbone of DNA [71]. The PDA liposomes **LP-18** and **LP-19** with positive charges were prepared by using primary and quaternary amine modified diacetylene monomers (Figure 1.16). On addition of the nucleic acids, amplified by common polymerase chain reaction (PCR), the color of polymerized liposomes **LP-18** underwent a transition from blue to red. Interestingly, primary



LP-18 **LP-19**

Figure 1.16 Polymeric PDA vesicles prepared from primary and quaternary amine receptor modified diacetylene monomers.

amine-functionalized **LP-18** displayed higher sensitivity than those containing quaternary amine-functionalized **LP-19**.

The main sensing strategy is based on the nonspecific ionic interaction with the positively charged PDA liposome. A simple purification step is required before the detection of DNA; this limits the detecting utility of this system in the aspect of its simplicity. Together with the above example, several other PDA liposome based sensors have been utilized to detect other important bioanalytes, including cations, antibodies, influenza virus, human serum albumin, carbonic anhydrase, *E. coli*, bacterial pore-forming toxin, thrombin, melamine, lectin, heparin, and pathogenic agents [50, 56–58, 72].

1.5 Boronic Acid Receptors for Diol-Containing Bioanalytes

Boronic acids receptors bind with diol units in aqueous solution to form cyclic boronate esters. Receptor design often uses the pK_a drop observed on addition of saccharides to boronic acids; the acidity of the boronic acids is enhanced when 1,2-, 1,3-, or 1,4-diols reversibly react with them to form cyclic boronic esters as five, six, or seven membered rings. This fundamental interaction is still of central importance in the construction of novel sensors for diol-containing (bio)analytes. A number of excellent review articles have been published in the past 2 years on boronic acid sensors [73–76]. Here we describe recent studies of innovative power in this field, such as high glucose selectivity, molecular logic with sugars, saccharide sensing at the few-molecule level of a reporter dye and drug delivery.

Glucose plays a dominant role in metabolic processes. For example, control of blood glucose concentration is of central importance for patients suffering

from diabetes mellitus. Thus, there is a strong clinical need for accurate glucose monitoring [75, 76]. Many synthetic boronic acid probes have been developed to selectively detect glucose at physiological concentrations [73, 74]. To develop a successful *in vivo* boronic acid-based glucose sensor, an important criterion that must be met is the preferential binding of glucose over other physiologically significant monosaccharides, such as fructose and ribose derivatives. This remains still a great challenge because most organic boronic acids display higher binding affinities against fructose [73, 75].

The groups of Jiang and James have recently developed a ratiometric fluorescent chemosensor based on an amphiphilic monoboronic acid that is highly selective and sensitive for glucose in aqueous solution [77]. The presence of glucose leads to pyrene excimer emission, while its monomer emission remains unchanged. In contrast, fructose results only in a modest enhancement of the monomer emission (Figure 1.17).

A sensor can also be interpreted as molecular switch using Boolean algebra. Prasanna de Silva *et al*. showed for the first time that molecular fluorescent probes for ions can function as logic gates [78]. Till now, fascinating molecular digital analysis with receptors and carbohydrates, oligonucleotides, oligopeptides, proteins, and metal ions have been shown [79–81]. First applications of these molecular logic gates can be found in the design of smart materials, in the delivery/activation of drugs, and in clinical diagnostics [82]. However, only very few chemical logic studies exist with boronic acids as saccharide receptors [83–85]. In 2012, we described a two-component saccharide probe with logic capability [84]. The combination of a boronic acid-appended viologen and perylene diimide was able to perform a complementary implication/not implication logic function (Figure 1.18). Fluorescence quenching and recovery with fructose was analyzed with fluorescence correlation spectroscopy on the level of a few molecules of the reporting dye. The study was highlighted as JACS Spotlight in 2012 [86].

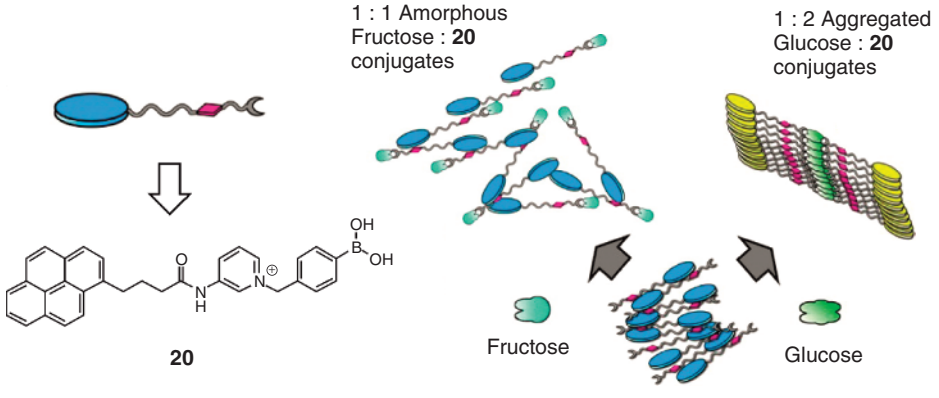


Figure 1.17 Amphiphilic monoboronic acid that is highly selective and sensitive for glucose. (Adapted and reproduced with permission from Ref. [77]. Reproduced with permission of American Chemical Society (United States).)

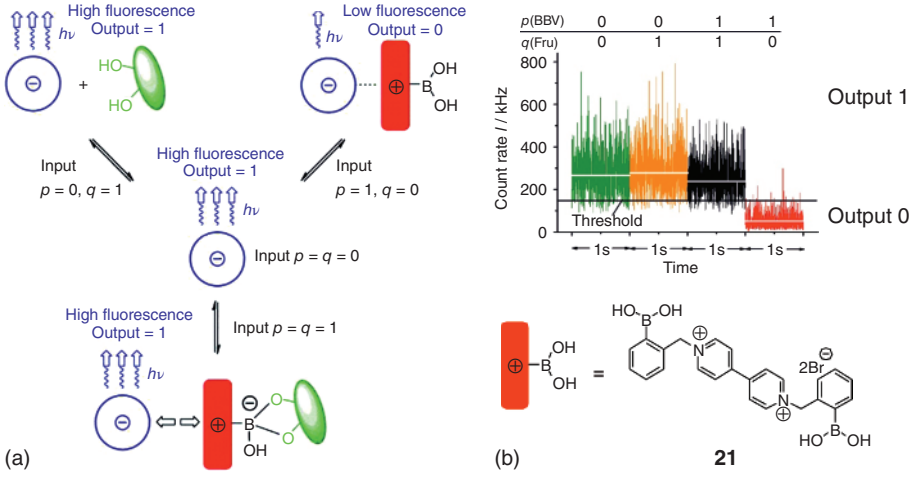


Figure 1.18 (a) A two-component saccharide probe performs IMP logic by an allosteric indicator displacement assay (AIDA). (Blue circle: fluorescent dye 1,6,7,12-tetrakis(4-sulfonylphenoxy)-*N*,*N*′-(2,6-diisopropylphenyl)perylene-3,4:9,10-tetracarboxidiimide (WS-PDI); red rectangle: saccharide receptor and quencher Bis-boronic acid appended Benzyl Viologen **21** (BBV); green ellipse: fructose, Fru.) (b) IMP logic gate via fluorescence transients in a confocal microscope from WS-PDI and the inputs p(BBV) and q(Fru): green (input 0,0), orange (0,1), black (1,1), and red (1,0). Truth table of IMP is also shown. (Adapted and reproduced with permission from Ref. [84]. Reproduced with permission of American Chemical Society (United States).)

Boronic acids can also be used for triggered drug delivery. A remarkable example was recently shown by the group of Kataoka [87]. Therapeutics based on small interfering RNA (siRNA) offer an attractive clinical option because of its ability to silence genes in a highly sequence-specific manner. siRNA was encapsulated by a phenylboronate-functionalized polyion complex (PIC) micelle. It binds to the phenylboronate via ribose of the siRNA thereby stabilizing the complex under conditions equivalent to an extracellular environment. This complex is disrupted in response to the addition of ATP, at a concentration comparable to that inside cells.

1.6 Conclusion and Outlook

To conclude, we have described promising examples for the recognition of important bioanalytes such as PPi, NTP, carbohydrates, LPS, and nucleic acids. Their potential application in biological cell staining, drug delivery, and molecular logic functions has also been mentioned.

Metal complex receptors with chromogenic or luminescent motif provide useful signals to measure the detection process. In addition, metal complex strategies offer

great advantages for bioanalytes binding in pure aqueous media with improved solubility of the receptor. The attractive feature of the colorimetric PDA vesicle systems is that they do not require any complex detection method, but it is rather a single step ''mix and observe'' process [58]. An important challenge in this field is to develop reversible PDA sensors for the detection of bioanalytes. Until now, no reversible PDA sensors for chemical or biological analytes are available.

Boronic acids are the commonly used receptor system for carbohydrate recognition. The combination of a boronic acid-appended viologen receptor and reporter dye was able to perform a logic function with fructose. This work demonstrates ways by which Boolean logic can process information in the field of sugar diagnostics [86]. The ability of boronic acid receptors to function effectively in water for bioanalytes within physiological and environmental scenarios was well studied. In recent years, efforts have moved to a more biological direction with applications in drug delivery, carbohydrate biomarkers, and array analysis.

Future advances in recognition of bioanalytes will involve the development of new intelligent methods to improve the binding properties of receptors in water. Although some examples are available now, discrimination between bioanalytes has been rarely achieved, and thus it is a clear challenge for the future. Recently, fluorine containing materials have been applied for monitoring different biological events such as enzymatic activity, cell viability, and biological reactions [88]. Thus, the development of fluorinated probes and using ^{19}FNMR spectroscopy for the detection of bioanalytes is a challenging and potential task within physiological condition. We believe that this chapter will inspire new advancement in the research area of bioanalytes recognition and discovering the future of molecular sciences.

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