Fluorescent and colorimetric chemosensors for detection of nucleotides, FAD and NADH: highlighted research during 2004–2010

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Due to the biological importance of nucleotides and related species, such as XNP (where X = adenosine (A), uridine (U), cytidine (C), guanosine (G), and N = mono, di, tri), FAD and NADH, the development of optical probes for these molecules has recently been an active area of research. This tutorial review focuses on the contributions between 2004–2010 concerning the fluorescent or colorimetric sensors for these biomolecules, and is organized according to their target molecule’s structural classification.

Introduction

The development of molecular-recognition and sensing systems for biologically important anions has received considerable attention in recent years.1–6 Recognition and sensing of nucleotides has been an especially active area of research due to their biological significance. Of all the nucleosides and nucleotides, the recognition of ATP and GTP is the most popular because ATP is not only a universal energy source but is also an extracellular signaling mediator in many biological processes,7 and GTP is involved in RNA synthesis, citric acid cycle, and acts as an energy source for protein synthesis.8

In addition to ATP and GTP, other nucleoside polyphosphates, such as adenosine monophosphate (AMP), adenosine diphosphate (ADP) and guanosine monophosphate (GMP), also play pivotal roles in various physiological events. For example, AMP and ADP are important for their roles in bioenergetics, metabolism, and transfer of genetic information, and GMP acts as an intermediate in the synthesis of nucleic acids and plays important roles in several metabolic processes.9

On the other hand, uridine triphosphate (UTP) and uridine diphosphate (UDP), the key building blocks for RNA synthesis and active in glycotransfer pathways, are widespread in living cells and play pivotal roles in various biological events.10

Thymidine nucleotides, including thymidine-monophosphate (TMP), -diphosphate (TDP) and -triphosphate (TTP), which are synthesised from thymidine in vivo, are essential building blocks in DNA replication and cell division.11 Imbalances in the cellular level of thymidine-triphosphate have been implicated in the development of diseases characterized by defects in the repair or replication of mitochondrial DNA (mtDNA).

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Besides these nucleosides and nucleotides, FAD (flavin adenine dinucleotide), NADH (nicotinamide adenine dinucleotide reduced form) and NAD$^+$ (nicotinamide adenine dinucleotide) are also very important biological molecules (Fig. 1). FAD is an important coenzyme in many biological systems, and is involved in numerous redox processes of metabolic reactions and biological electron transport. It has been reported that the human white blood cells (eosinophils) have a considerable amount of FAD in their granules, and the FAD in eosinophils plays an important role in redox reactions to generate hydrogen peroxide or superoxide.\textsuperscript{12}

As the key central charge carriers in living cells, the NADH/NAD$^+$ couple transfers hydrogen atoms and electrons from one metabolite to another in many cellular redox reactions and is used as the cofactor in >300 dehydrogenase enzymatic reactions.\textsuperscript{13}

Due to the significant roles of these molecules in biological systems, considerable effort has been devoted to develop detection methods for them. Although several methods are currently available for their determination, some problems still arise due to the operation of sophisticated chromatographic instrumentation or a combination of the procedure and its quantitative reproducibility. Fluorescent sensors are powerful tools to monitor \textit{in vitro} and/or \textit{in vivo} biologically relevant species because of the simplicity and high sensitivity of fluorescence.\textsuperscript{14} Hence, the development of fluorescent and colorimetric chemosensors for their detection has attracted significant attention over the past few years.\textsuperscript{15}

Even though some literature reviews have covered the sensors of nucleotides in a single chapter,\textsuperscript{1,16,17} quite a number of important recent contributions are missing in those reviews. In this review, we will focus on the fluorescent and colorimetric chemosensors developed for the detection of these biologically important molecules, including nucleotides, FAD and NADH, since 2004. The receptors are classified according to their target molecule’s structural classifications. In this review, we hope to provide a general overview of the design and applications of these chemosensors.

**Colorimetric and fluorescent methods to detect ATP**

**Chemosensors bearing imidazolium or quaternary ammonium groups as the binding motif**

Inouye and coworkers reported a pyrenophane system having large neutral cavities and exhibiting eximer emissions due to the proximity of the pyrene units (Fig. 2).\textsuperscript{18} Probe 1 preferred to form complexes with nucleotide triphosphates, especially GTP and ATP, rather than with di- or monophosphates. When 1 was titrated with ATP in water, the UV-vis spectra changed in a hypochromic way according to \(\pi\)-stacking coordination, and the association constant was determined as \(1.0 \times 10^6 \text{ M}^{-1}\). As shown in Fig. 2, the selective binding with nucleotide triphosphates was explained on the basis of the exact fit of the nucleotide triphosphates within the cavity of the

![Fig. 1 Structures of nucleotides, FAD and NADH.](image1)

**Juyoung Yoon**

Juyoung Yoon was born in Pusan, Korea in 1964. He received his PhD (1994) from The Ohio State University. After completing postdoctoral research at UCLA and at Scripps Research Institute, he joined the faculty at Silla University in 1998. In 2002, he moved to Ewha Womans University, where he is currently a Professor of Department of Chemistry and Nano Science and Department of Bioinspired Science. His research interests include investigations of fluorescent chemosensors, molecular recognition and organo EL materials.

![Fig. 2 The proposed mechanisms of multipoint recognition of 1 for nucleotide.](image2)
pyrenophane, which was aided by the synergistic coordinative interaction.

On the other hand, Yoon et al. investigated a pincer-like benzene-bridged sensor 2 with a pyrene excimer as a signal source and imidazolium as a phosphate anion receptor for ATP sensing (Fig. 3). At pH 7.4, a unique switch of excimer vs. monomer pyrene fluorescence of 2 was observed in the presence of ATP due to the characteristic sandwich π–π stacking of pyrene–adenine–pyrene. On the other hand, GTP, CTP, UTP and TTP can interact only from the outside with the stacked pyrene–pyrene dimer of 2. A high selectivity was achieved by the larger fluorescent intensity ratio of monomer-to-eximer for 2 upon binding with ATP ($I_{575}/I_{487}$), and the association constant for ATP was calculated as $1.03 \times 10^5 \text{ M}^{-1}$. The biological application of 2 in cultured cells (HeLa cells) was further investigated, and chemosensor 2 was also applied to establish a ratiometric fluorescence assay for ATP-relevant enzyme activity.

Duan’s group recently reported aminonaphthalimide imidazolium podands with 1,3,5-tris(bromomethyl)-2,4-dimethylbenzene as the six hydrogen-bond donors, which functioned as chemosensors for selectively sensing nucleoside polyphosphates through a “turn-on” manner. Sensor 3 exhibited a selective “turn-on” fluorescent property for ADP over other ribonucleotide polyphosphates. Sensor 4 exhibited a similar fluorescent property for ATP, GTP and UTP (Fig. 4). The Hill-plot profile of the titration curve suggested a 1 : 2 stoichiometry of the host–guest complexation species, with the association constant $K_a = 8.75$. Fluorescence enhancement of 4 was observed when stained HeLa cells were further incubated with ATP (0.4 mM).

Atilgan and Akkaya reported a calixpyridinium–pyranine complex as a selective anion sensing assembly via the indicator displacement strategy (Fig. 5). Strong binding interaction between the positively charged receptor (calixpyridinium tetracation, 5) and the fluorescent pH indicator (pyranine, 6; 8-hydroxy-1,3,6-pyrenetrisulfonate) requires an anionic guest of comparable negative charge for effective indicator displacement. Thus, the selective and sensitive signaling of ATP was achieved in aqueous solutions. The displacement assays were conducted in MOPS buffer (0.1 M, pH 7.5). Addition of ATP up to 1.0 mM resulted in significant displacement of the dye with a concomitant increase in the emission intensity of the pyranine at 520 nm. The dissociation free energy for the ATP–5 complex was calculated as 6.1 kcal mol$^{-1}$.

Ramaiah’s group developed a pyridinium–anthracene cyclodiphane system which can discriminate ATP from other nucleosides, nucleotides, and phosphate anions under physiological pH conditions (Fig. 6). The uniqueness of this system was that it complexed with ATP through synergistic effects of electrostatic and π–π stacking interactions in a cavity. With increasing concentrations of ATP, 7 showed a decrease in the absorption corresponding to the anthracene moiety. Benesi–Hildebrand analysis of the absorption changes showed a 1 : 1 stoichiometry for the complex formed between 7 and ATP, with a binding constant of $4040 \pm 140 \text{ M}^{-1}$ in buffer solution.

Zong et al. reported a l-arginine derivative (8), and its interaction with ATP in aqueous solutions (Fig. 6). The most interesting feature in this system is the quenching induced by the addition of ATP at pH values <4.0; the anion-induced fluorescence spectra changes of 8 were tested in aqueous solutions at pH = 3.0. Compound 8 displays a chelation-enhanced fluorescence quenching (CHEQ) effect for ATP with an association constant $K_a = 3.49 \times 10^5 \text{ M}^{-1}$, and relatively small CHEQ effects for ADP and AMP. Almost no fluorescent changes are observed for other anions.
A two-input NAND logic gate was presented on the basis of this system.

Moreno-Corral and Lara have synthesized acridine and anthraquinone containing cyclophanes based on the (S),(S)-(+)-tetrandrine moiety and have studied their binding interactions with nucleotides and amino acids (Fig. 7).\textsuperscript{24} Utilizing the ion pairing and hydrophobic interactions, both of these cyclophanes (10 and 11) were found to interact with various nucleotides with increasing guest charge in the series ATP > ADP > AMP. The association constant of ATP, ADP and AMP to the ion pairing and hydrophobic interactions, both of these complexes of ATP, ADP and AMP, respectively.

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Král’s group reported tetrabrucin–porphyrin as a sensor, which showed the condition of multiple binding modes leading to selectivity for ATP over ADP and AMP under physiological conditions (Fig. 8).\textsuperscript{25} Because of the aggregation of 12 in water, all measurements were conducted in a methanol–HEPES buffer (1 : 1, v/v). Addition of nucleotides (AMP, ADP, ATP) caused a decline in the absorbance maximum of 12. The calculated values of the stability constants for 1 : 1 complexes of 12 and nucleotides were 64000, 6700 and 2400 M\textsuperscript{-1} for ATP, ADP and AMP, respectively.

Fig. 7 Structures of fluorescent probes 10 and 11.

Chemosensors based on hydrogen bond interactions: azacrown, amide and urea derivatives

In 2006, Bazzicalupi and Bencini \textit{et al.} reported a new phenanthroline-containing macrocycle (13) which could selectively recognize and sense ATP among triphosphate nucleotides at pH 6 (Fig. 9).\textsuperscript{26} Addition of increasing amounts of ATP to a solution of 13 produced a linear decrease of the fluorescence emission of 13 at 365 nm, and the fluorescence of 13 was completely quenched in the presence of 1 eq. of ATP. ADP, AMP, GTP and TTP produced a slight decrease of the fluorescence emission, while a larger fluorescence emission decrease of ca. 30% was observed in the presence of an excess of CTP. MD calculations of the binding mode showed that the bent conformation of the nucleotides allowed the formation of hydrogen bonding interactions of the γ-phosphate groups of both ATP and CTP with the benzyl nitrogens. However, the γ-phosphate of CTP is pushed away from the benzyl ammonium groups and cannot provide hydrogen bonding interactions with them, while the γ-phosphate of ATP can provide a couple of strong hydrogen bonds with these nitrogens, which leads to the observed selectivity for ATP.

Bazzicalupi and Bencini \textit{et al.} extended their work and reported a new phenanthroline derivative (14) and compared its properties with 13 (Fig. 9).\textsuperscript{27} It was mentioned that the fluorescent emission of both receptors depended on the protonation state of the polyamine chain. However, only receptor 13 was large enough to allow the π-pairing of nucleobases with phenanthroline in the order: ATP ≥ CTP ≥ TTP ≥ GTP. Despite the bent conformation assumed by nucleotides, the nucleobases cannot achieve an appropriate disposition to give π-stacking with the phenanthroline of 14. Fluorescence emission measurements at different pH values showed that 13 was also able to ratiometrically sense ATP in a narrow pH range (4.5–7). This suggested that the quenching effect was likely related to a proton-transfer process from an ammonium group to the anionic phosphate chain.

Kosterin and co-workers reported a new flavonol fluorescence probe (15) which could sense ATP in an aqueous medium (Fig. 9).\textsuperscript{28} probe 15 displayed a band with a maximum fluorescence emission of ~400–410 nm, and the presence of ATP at pH 7.4 caused a red-shift of 60 nm to the new maximum of 470–480 nm, with a total increase of emission intensity. However, interaction with ATP caused only an increase of intensity in the fluorescence spectra of probe 15, while band shape and the position of maximum fluorescence underwent small changes. The dynamics of the observed effect were studied in mitochondria. The registered phenomenon allowed the quantitative evaluation of ATP concentration in the range of 10\textsuperscript{-3}–10\textsuperscript{-5} M. In contrast to ATP, other

Fig. 9 Structures of chemosensors 13, 14 and 15.
nucleoside phosphates did not give a new band in the excitation spectra of probe 15.

The tripodal sensor molecules 16–23, based on 1,3,5-triaminomethyl-2,4,6-triethylbenzene, which can form arrays of six hydrogen-bond donors, were embedded in hydrophilic polyurethane matrices (Fig. 10).29 These tripodal sensors were reported to provide turn-on effects for various phosphate ions including AMP and ATP. A 1 : 1 complex with $C_3$ symmetry, in which all three receptor arms are equally involved in the formation of the complex, was suggested in the binding of anions by 16–23. Sensors 17–19 and 21–23 were embedded in hydrophilic polyurethane matrices to circumvent the fact that 17–19 and 21–23 were not soluble in water. In the array, water (control), serum, and serum samples with added anions (phosphate, pyrophosphate, AMP or ATP) were applied. Because of its intrinsic anion content, the serum itself showed 15% fluorescence quenching of 24, this sensing can be conducted in aqueous solutions with sensitivity on the sub-micromolar level.

**Chemosensors using the zinc complex as the binding motif**

Due to the biological importance of phosphate anion species, molecular recognition and fluorescence sensing by using a variety of artificial receptors consisting of a $Zn^{2+}$-complex as a common binding motif has been reported,31,32 and in recent years the sensing systems using $Zn^{2+}$ complexes as the binding motif have become an indispensable part of ATP sensor designs.

In 2004, Hamachi’s group reported two fluorescent chemo-sensors bearing two zinc ions coordinated to distinct dipicolylamine (Dpa) sites (Fig. 11).33 It is reported that 25 and 26 showed a high selectivity for phosphate derivatives among the various anions in aqueous solution. They showed a stronger binding affinity toward the pyrophosphate derivatives such as ATP and ADP compared to the monophosphate species. The apparent binding constant ($K_{app}$) of 25 for ATP and ADP was $>10^7 M^{-1}$, and the $K_{app}$ of 26 was reported as $4.0 \times 10^5 M^{-1}$ (for ATP) and $1.6 \times 10^5 M^{-1}$ (for ADP).

Since 26 could not discriminate for a specific phosphate species among the phosphate family, including phosphorylated peptides in aqueous solution, the authors conducted their work using a new system, in which 26 and 27 (Fig. 12) were embedded in a glycosylated amino acid type of hydrogelator (TSH 1).34 Upon ATP addition, the emission of 26 at 435 nm increased by 2.6-fold in fluorescence intensity, with a binding constant of $>10^7 M^{-1}$. After the receptor 27 was immobilized in the supramolecular hydrogel, the addition of various anions led to three patterns of fluorescence changes: the emission intensity of 27 (at 512 nm) increased with the emission blue-shift for PhP, and the intensity decreased with the emission red-shifts for ATP, phosphate and phospho-Tyr, whereas non-phosphate anions did not induce any fluorescence change. It is important to note that the hydrophobic domain of the present supramolecular hydrogel is crucial for the guest-induced fluorescence change.

In 2006, Ojida and Hamachi developed the dual-emission chemosensors 28 and 29 based on acridine for nucleoside PP derivatives under neutral aqueous conditions (Fig. 13).35 Upon addition of ATP to 28 at pH 7.2, the emission maximum
at 468 nm immediately decreased and blue-shifted to 441 nm. In the case of 29, a seesaw-type spectral change was observed upon ATP binding and the original green fluorescence emission of 29 turned blue. Strong binding affinities of ATP at 7.6 × 10^6 M⁻¹ and 5.3 × 10^6 M⁻¹ were observed for 28 and 29, respectively. As shown in Fig. 13, a unique shift in emission wavelength was ascribed to the dissociation of the first Zn²⁺ ion from the acridine nitrogen atom as a result of the formation of the binuclear Zn²⁺ complex. Furthermore, the chemosensor was successful in some biological assays, such as fluorescence monitoring of apyrase-catalyzed hydrolysis of nucleoside PPs and glycosyl transfer catalyzed by glycosyltransferase.

In the same year, Ojida and Hamachi reported a new chemosensor (30), which is comprised of a xanthene fluorophore that bears two 2,2'-dipicolylamine (Dpa)-Zn²⁺ moieties as a phosphate-binding site for ATP detection (Fig. 14). Under neutral aqueous conditions, the changes in excitation with increasing concentration of ATP occur at three wavelengths (322, 360, 407 nm) with two isomission points. Chemosensor 30 showed strong affinities towards nucleoside pyrophosphates such as ATP (K_a = 4.5 × 10^5 M⁻¹), GTP (K_a = 1.0 × 10^6 M⁻¹), and ADP (K_a = 2.8 × 10^5 M⁻¹), whereas binding was relatively weak for the HPO₄²⁻ ion and monophosphate derivatives such as AMP.

Das et al. reported a chromogenic metal complex (31) which could selectively bind to ATP in contrast to other biologically important anions such as ADP, AMP, PPI, or phosphate, under aqueous conditions (Fig. 14). The zinc dipicolylamine unit in 31 acts as the receptor fragment for ATP, whereas the dimethylamino-phenylazo group acts as the signaling group for reporting the binding. Upon addition of ATP at pH 7.2, the absorption maximum shifted from 463 nm to 484 nm, with a color change from pale yellow to light pink. A small red shift of 8 nm was observed with ADP and no change in absorption spectra was observed upon addition of AMP, PPI or H₂PO₄⁻. Furthermore, this receptor molecule could be used as a colorimetric staining agent for yeast (Saccharomyces cerevisiae) cells, and staining could be viewed by simple light microscopy.

The same group further explored the possibility of using this receptor molecule as a colorimetric staining agent for prokaryotic microbes, and for studying cellular growth kinetics. 31 was used as a viable staining agent for both prokaryotes and eukaryotes through binding to ATP produced in situ during metabolic processes, and could be used for probing the growth profiles of microbes and cells.

Hamachi’s group reported other ratiometric chemosensors (32 and 33) for ATP based on binding-induced modulation of fluorescence resonance energy transfer (FRET), coupled with a turn-on fluorescence-sensing mechanism (Fig. 15). Upon the addition of ATP under aqueous conditions at pH 7.2, excitation of the coumarin unit at 341 nm induced a clear seesaw-type dual-emission change, suggesting the recovery of FRET from the coumarin to the xanthene. The FRET efficiencies of the ATP bound complexes of 32 and 33 were calculated as 76% and 83%, respectively. Curve-fitting analysis provided an affinity constant (K_{app}) of 2.9 × 10^6 M⁻¹ for ATP with 1 : 1 binding stoichiometry. During the titration of 33 with ATP, a decrease in the coumarin fluorescence and a concomitant large increase in the xanthene fluorescence were observed. These chemosensors were used for real-time fluorescence monitoring of enzyme reactions which was further demonstrated in a fluorescence-imaging study of the nucleoside polyphosphates inside living cells, such as HEK293 and NIH3T3 cells.

A dimetallic coordination fluorescent chemosensor (34) for ATP was improved by eliminating the intrinsic background fluorescence of the sensor by simply incorporating pyrocatechol violet as a quencher (Fig. 16). This ensemble could easily detect <0.5 μM of ATP and could discriminate between ATP, ADP and various other anions. The addition of pyrocatechol violet could induce a reduction in fluorescence of 34 by forming a 1 : 1 binding mode with an association constant of 2.89 × 10^3 M⁻¹. In this sensor ensemble, the presence of ATP resulted in a large enhancement of fluorescence due to
Zn$^{2+}$ is coordinated by four nitrogen atoms of the macrocycle increase in the emission of the monomer. As shown in Fig. 17, in the emission of the anthracene excimer and a remarkable for ATP in neutral aqueous solution by exhibiting a decrease in fluorescence at 535 nm (Fig. 17). Complex 35-Zn$^{2+}$ exhibited an excellent selectivity for ATP in neutral aqueous solution by exhibiting a decrease in the emission of the anthracene excimer and a remarkable increase in the emission of the monomer. As shown in Fig. 17, Zn$^{2+}$ is coordinated by four nitrogen atoms of the macrocycle and two amino groups on the pendent arms, which resulted in proximity between the two fluorophores. When 10 equiv. of PPi or ATP was added at pH 7.4, the excimer emission of 35-Zn$^{2+}$ was quenched, whereas monomer emission increased 3.8-fold and 13.7-fold, respectively.

A naphthalimide fluorescent chemosensor (36) for ATP, based on a Zn$^{2+}$–DPA complex unit, was reported by Mohr and co-workers (Fig. 18). A significant increase in fluorescence at 535 nm (~2.3 fold) at pH 7.4 was observed with increasing concentrations of ATP, even as low as 1 μM. Among the various anionic species, only ADP displayed a comparable binding constant to ATP. In 36, complexation of the DPA unit with Zn$^{2+}$ presents itself with two effects: first, a decrease of PET which leads to an increase in fluorescence and secondly, a de-excitation channel due to the interaction between Zn$^{2+}$ and the aromatic NH group as shown in Fig. 18. Binding of ATP to the Zn$^{2+}$–DPA unit decreases the strength of the Zn$^{2+}$–NH interaction, resulting in a significant increase in fluorescence.

Chemosensors using the other metal ions or metal complexes as the binding motif

In recent years, ATP sensors utilizing other metal ions or metal complexes, such as ytterbium (Yb$^{3+}$), ruthenium (Ru$^{2+}$), europium (Eu$^{3+}$), terbium (Tb$^{3+}$), yttrium (Y$^{3+}$), manganese (Mn$^{2+}$) or cobalt (Co$^{2+}$), as the binding motif have also been studied. Some of these complexes possess coordinatively unsaturated central metal ions and, therefore, have vacant coordination sites for the incoming anions and ensure the optical changes.

Xu et al. reported a coordinatively unsaturated lanthanide complex (37), and demonstrated that not only phosphate and pyrophosphate, but also DNA and RNA could be distinguished by visible absorption or fluorescence spectra of 37 (Fig. 18). In tris-HCl (pH = 7.4) buffered solution of 37, AMP and ADP induced optical responses (UV) similar to one-molecule of HPO$_4^{2-}$ and PiO$_4^{2-}$, respectively. As for ATP, a strong new absorption at long wavelength was observed. The stability constant for the complex of ATP with 37 was determined to be (2.2 ± 0.3) × 10$^4$ M$^{-1}$ with a detection limit of 9.0 × 10$^{-7}$ M. The fluorescence intensity of 37 was enhanced by addition of ATP, ADP or AMP.

Yoon et al. reported a new fluorescein derivative bearing two (2-aminoethyl)bis(2-pyridylmethyl)amine groups for the detection of PPi and ATP in 100% aqueous solution (Fig. 18). Chemosensor 38, in the presence of Mn$^{2+}$ (2.5 equiv.), displayed significant selective fluorescent enhancements with PPi and ATP at pH 7.4, and a relatively smaller fluorescent enhancement with ADP. The association constants for PPi, ATP and ADP were calculated as 4.2 × 10$^5$, 3.5 × 10$^4$ and 1.3 × 10$^5$ M$^{-1}$, respectively. It is worth mentioning that discrimination between PPi and H$_2$PO$_4^-$ was excellent in 100% aqueous solution, and selectivity for ATP over ADP and AMP was achieved in this system.

Metallohelical triangles consisting of chromophore units and hydrogen bonding trigger sites were prepared by Duan’s group for the selective detection of ATP in aqueous media (Fig. 19). The cobalt(i)-based helical triangles were synthesized by adding NH$_4$PF$_6$ into a methanol solution of ligands.
(L-39, L-40) and Co(NO$_3$)$_2$·6H$_2$O. These cobalt(II)-based helical triangles of 39 and 40 exhibited almost the same UV-vis spectral changes upon the addition of ATP, with a significant absorbance increase at 300 nm and an obvious absorbance decrease at 380 nm. They could form 1 : 1 stoichiometric complexes with ATP, and the association constants ($\log K_{assoc}$) were calculated as 2.55 ± 0.03 and 3.86 ± 0.03, respectively.

**Chemosensors linked polymers or mesoporous materials**

Shinkai’s group reported a water-soluble cationic polythiophene derivative (41) which displayed colorimetric and fluorescent responses to ATP through electrostatic and hydrophobic cooperative interactions (Fig. 20). Upon adding increasing amounts of ATP to 41 in water, the absorption maximum was gradually red-shifted from 400 nm to 538 nm, with an observed dramatic color change from yellow to pink-red. After addition of an equimolar amount of AMP, ADP, UTP, carboxylate, phosphate, or triphosphate ions to aqueous solutions of 41, most of the solutions remained yellow with $\lambda_{\text{max}}$ 435 nm, except for those that contained ADP or UTP, which gave orange solutions. In addition, the quenching of fluorescence was much more effective in the presence of ATP (84%), with a detection limit of 10$^{-8}$ M.

Shi et al. extended the research on 42 and published their work as the first observation of colorimetric discrimination of 15 XNPs based on this single conjugated polymer probe (Fig. 20). Upon addition of XNPs at pH 7.4, the absorption maximum of 42 was red-shifted from 405 to 416 (AMP), 455 (ADP), 542 (ATP), 499 (UTP), 480 (TTP), 540 (GTP) and 469 nm (CTP), respectively. A linear discriminant analysis (LDA) and colorimetric method were used to classify the fifteen nucleotides. According to the color changes, a pink-purple group consists of ATP, ADP, UTP, TTP, GTP, GDP and GMP, whereas the remaining eight nucleotides constitute the second yellow-orange group. These discrepancies in color can be used as a colorimetric method to roughly distinguish certain XNPs, especially for ATP over ADP and AMP, and UTP over UDP and UMP.

Wang’s group reported the water-soluble complex of conjugated polymers/enzymatic substrates which can be utilized as a probe for continuous, sensitive and facile fluorescence assays for AXP (X = T, M, D) (Fig. 20). The emission maximum of 42 itself at pH 7.5 appeared at ~410 nm and no emission was observed from the 2,1,3-benzothiadiazole (BT) unit at 540 nm. Adding oppositely charged substrates (ATP) led to a significant quenching of the fluorene unit emission at 410 nm, and the appearance of the BT unit emission at 540 nm, followed by a shift in emission color from blue to green. This distinct shift in emission color was characteristic of the aggregation of the main chains of 42 with a detection limit of 40 nm. There was less quenching of the emission of 42 upon the addition of ADP or AMP, and also no emission from the BT unit was observed at 540 nm. This result showed that ADP and AMP, containing less negative charges in comparison to ATP, did not induce the aggregation of 42.

Amorós et al. reported the use of an anthrylmethylamino ligand (43) anchored to mesoporous silica UVM-7 materials for the fluorescence detection of ATP in aqueous media at pH 2.8 (Fig. 21). For the solid UNA(2-E)-C, addition of ATP resulted in a quenching of the anthracene emission, and a remarkably larger sensitivity for ATP was observed with $\log K = 4.78$. For the solid UNA(10-E)-C, besides the decrease of fluorescence at 415 nm, ATP addition resulted in an increase of a new emission band at ca. 500 nm. This new unstructured broad band, red-shifted with respect to the monomer band, can be assigned to the formation of excimers between neighboring anthracene groups. As shown in Fig. 21,
in the presence of ATP, the electrostatic repulsion would decrease and the anthracene groups would be in closer proximity, making the formation of excimers more probable.

A new sensing system via attaching different polyamine chains functionalized with an indole fluorophore to a boehmite matrix was reported by García-Espana et al. (Fig. 22).50 Steady-state fluorescence emission studies showed that these materials presented a very efficient sensing behavior for hydrogen ions, metal ions such as Cu²⁺ and Zn²⁺ and for the anionic nucleotides—ATP, ADP and AMP. All three materials showed a decrease in their fluorescence when increasing amounts of ATP were added, however 44 showed a smaller interaction than the other two. Among all the factors, the π-stacking between the electron-rich fused ring of indole and the adenine ring of the nucleotide should play important roles in the recognition. ADP and AMP showed slightly less interaction with the three materials.

Tao et al. developed a new fluorescent detection system for ATP, utilizing the ATP-mediated aggregation process of pyrene-appended boronic acid (47) on a polycation (48) (Fig. 23).51 Large spectral changes were observed in this system upon the addition of ATP and ADP, whereas a very small response was observed upon addition of AMP. With increasing ATP concentration in an aqueous polycation solution at pH = 10.2, a broad fluorescence band with an excimer emission maximum at 482 nm was intensified. This system could detect the concentrations of ATP in the range of 0.4 to 5 μM, with a detection limit of 0.1 μM.

**Fluorescent and colorimetric sensors for GTP**

Kim and Yoon et al. reported the water-soluble imidazolium anthracene derivative 49 (Fig. 24), which not only senses GTP by a chelation-enhanced fluorescence quenching (CHEQ) effect in 100% aqueous solution at pH 7.4, but also differentiates the structurally similar compounds ATP, ADP and AMP by a chelation-enhanced fluorescence (CHEF) effect for the first time.52 The guanine base in GTP acted as a fluorescence quencher, which resulted in the significant CHEQ effect, with a slight red-shift of 49 towards the addition of GTP. From the fluorescence titrations, the association constant for GTP is 8.7 × 10⁴ M⁻¹, which is ~6 times larger than that for ATP, and >100 times those for ADP, AMP, pyrophosphate, H₂PO₄⁻, F⁻ and Cl⁻. The NMR tests and the computed geometries of 49–GTP complex suggested additional interactions of the nucleic bases with the anthracene moiety of 49 in the T-shape.

Yoon’s group also reported a fluorescent cavitand derivative bearing four imidazolium groups as well as four pyrene groups as a fluorescent receptor for GTP (Fig. 24).53 In DMSO–HEPES (6 : 4, v/v), cavitand (50) displayed a large CHEQ (chelation enhanced fluorescence quenching) effect with GTP and relatively small CHEF effects for ATP, CTP and ADP. There were almost no fluorescent changes, even when 100 equiv. of pyrophosphate and H₂PO₄⁻ were added. From the fluorescent titrations, the association constants for GTP, ATP and CTP were calculated as 7.4 × 10³, 1.4 × 10⁴ and 7.7 × 10³ M⁻¹, respectively, which showed that the selectivity for GTP was >5-fold and 10-fold greater than the selectivity for ATP and CTP.

Wang and Chang reported the synthesis of a combinatorial benzimidazolium dye library and the discovery of the first turn-on fluorescent GTP sensor (51) (Fig. 25).54 Two structurally related compounds (51, 52) showed dramatically increased

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**Fig. 22** The structure of ligands 44, 45 and 46-based materials.

**Fig. 23** Structures of pyrene-appended boronic acid (47), polycation (48) and the ATP-mediated aggregation of 47 on 48.

**Fig. 24** Structures of chemosensors 49 and 50.

**Fig. 25** Structures of chemosensors 51–53 and HPTS.
fluorescence upon addition of GTP, while not responding to other nucleotides. To fully check the selectivity of the two compound hits, all the nucleosides and nucleotides were tested systematically in a 96-well plate. High selectivity of both 51 and 52 for only GTP was clearly exhibited without any obvious cross-response to any of other nucleotides or nucleosides. Upon addition of GTP to 51, a red shift for both \( \lambda_{ex} \) (from 450 to 480 nm) and \( \lambda_{em} \) (from 520 to 540 nm) was observed. When excited at 480 nm, an \( \sim \) 80-fold fluorescence enhancement at 540 nm was observed only for GTP, while only small changes were observed for all other analytes.

A highly sensitive and selective fluorescence assay for GTP, made possible through beneficial properties of 53 and the fluorescence indicator, HPTS, was reported by Ramaiah’s group (Fig. 25).55 This assay successfully discriminates GTP from ATP and other nucleotides and nucleosides through an on–off–on fluorescence mechanism, with a visual change in fluorescence intensity. Additions of receptor 53 to a solution of indicator (HPTS) in buffer resulted in a regular decrease in the absorbance and fluorescence quenching of HPTS centered at 512 nm. A 1 : 1 stoichiometry for the complex \([\text{GTP} - \text{HPTS}] \) by gradual addition of GTP, and a regular enhancement in fluorescence intensity corresponding from ATP and other nucleotides and nucleosides through an on–off–on fluorescence mechanism, with a visual change in fluorescence intensity. Additions of receptor 53 to a solution of indicator (HPTS) in buffer resulted in a regular decrease in the absorbance and fluorescence quenching of HPTS centered at 512 nm. A 1 : 1 stoichiometry for the complex \([\text{GTP} - \text{HPTS}] \) by gradual addition of GTP, and a regular enhancement in fluorescence intensity corresponding to HPTS at 512 nm were observed. The competitive displacement of the indicator by various analytes was found to be in the order: GTP(buffer) \( \approx \) GTP(biofluid) ITP \( \gg \) ATP \( \approx \) UDP \( \approx \) ADP \( \approx \) AMP \( \approx \) Ade.

Fluorescent and colorimetric sensors for UTP and UDP

Hamachi and co-workers continued their work on 26 as the chemosensor for the glycosyltransferase assay (Fig. 13).56 Under aqueous neutral conditions, 26 displayed a blue fluorescence at 415 nm, the intensity of which increased by \( \sim 200\% \) upon the addition of a micromolar range of UDP. The apparent binding constant \( (K_{app}) \) of 25 to UDP was estimated to be \( 3.8 \times 10^5 \text{ M}^{-1} \), and the binding of 25 to UDP-Gal was weaker by about two orders of magnitude \( (4.4 \times 10^3 \text{ M}^{-1}) \). The difference in the sensitivity of 25 gave a concentration window suitable for quantitatively detecting UDP in the range of 1 to 10 \( \mu \text{M} \), without interference from UDP-Gal. It was the first supramolecular method involving application of a chemosensor for the glycosyltransferase assay, with a reliable output, even under multistate enzymatic conditions.

Yoon’s group recently uncovered a water-soluble, “Off–On” type fluorescence sensor based on a perylene-bis(2-pyridylmethyl)amine-Zn platform (Fig. 26).57 This sensor exhibited high selectivity for UTP and UDP relative to other phosphate derivatives in aqueous solution at physiological pH. In the presence of UTP and UDP, 54 displayed selective fluorescence enhancements at 550 nm. The association constants for UTP and UDP were calculated as \( 6.0 \times 10^5 \text{ M}^{-1} \) and \( 1.1 \times 10^5 \text{ M}^{-1} \), respectively. As shown in Fig. 26, a predominant mononuclear \( \text{Zn}^{2+} \) complex was speculated, due to the weak fluorescence in aqueous buffer. The addition of UDP or UTP induced a second \( \text{Zn}^{2+} \) complexation to form the UDP-54 or UTP-54 complex. 54 was successfully applied to monitor both steps of glycosylation processes in a convenient and quantitative manner.

König et al. reported two pyrene labelled \( \text{Zn}^{2+} \)-cyclen (55) and \( \text{bis-Zn}^{2+} \)-bis-cyclen (56) complexes (Fig. 27).58 The addition of 0.5 equivalents of UDP, UTP, TTP or PPI to 55 in aqueous HEPES buffer \((\text{pH} = 7.4)\) induced a strong increase in pyrene excimer emission, observable with the unaided eyes. A 2 : 1 aggregate of 55 and the respective nucleotide or pyrophosphate was suggested. As shown in Fig. 27, UTP or TTP can act as a bidentate guest, templating the assembly of two complexes (55). The close proximity of the two pyrene moieties resulted in an increased excimer emission intensity. Compound 56 showed a different selectivity pattern and increased excimer emission was observed in the presence of UTP, UDP, IDP or ITP.

Fluorescent and colorimetric sensors for TTP

Hong and co-workers reported an ensemble sensor system that used the FRET-pair Flrpic–\( \text{Zn}^{2+} \)-DPA, an acceptor with preference for the triphosphate moiety, and mCP–\( \text{Zn}^{2+} \)-cyclen,
that of other nucleotide ensemble systems.60 It exhibited a stronger affinity and "Off–On" response to the adenosine guest of ~60-fold over all other nucleobases. Furthermore, a remarkably selective potentiometric response was attained for 5'-AMP over 5'-GMP, 5'-CMP and 5'-UMP by using an ion-selective electrode with a PVC-supported solvent polymeric membrane. This indicated that recognition of water soluble nucleotide guests through the membrane–water interface was possible.

Lin et al. reported a Cu-coordinated complex based on a 7-membered amide cycle, and its binding ability with nucleotides (AMP, ADP, ATP) has been studied by UV–vis spectrum in DMSO/H2O (9 : 1).62 With the addition of AMP, the absorbance band of 62 (Fig. 30) at ~325 nm gradually increases, while the intensity of the absorbance band at ~413 nm gradually decreases. The new absorption band developed at 420 nm with the addition of ADP, and the addition of ATP also induced a similar spectral change of 62. The stability constant of 62 with AMP is 39 754 ± 5637 M⁻¹, and the binding ability of nucleotides with 62 was in the order: AMP > ADP > ATP. The binding ability of nucleotides with 62 was influenced by the chain length of nucleotides, therefore the strongest chain among studied nucleotides, AMP, showed the stronger binding ability and stacked well with 62.

Fluorescent and colorimetric sensors for GMP

Fabbrizzi et al. demonstrated that the dimetallic cryptate [Cu₄(63)]⁺ selectively recognized guanosine monophosphate with respect to other nucleoside monophosphates (NMPs) in a MeOH/water solution at pH 7 (Fig. 31).63 Recognition was efficiently signaled through the displacement of the indicator.
such as natural FMN cyclase. The kinetics data for FAD splitting by 65 were calculated to be $k_{cat} = (6.0 \pm 0.2) \times 10^{-3}$ s$^{-1}$ and $K_m = (1.0 \pm 0.1) \times 10^{-5}$ M$^{-1}$. 65 enabled the first selective fluorescent detection of FAD by a splitting mechanism and sensing of FMN by a PET mechanism with $k_{cat} = (2.8 \pm 0.1) \times 10^{-3}$ s$^{-1}$ and $K_m < 1 \times 10^{-5}$ M$^{-1}$. 66 was also successfully applied to a real-time fluorescent assay of various enzymatic reactions related to flavin conversions.

**Fluorescent and colorimetric sensors for NADH**

Willner et al. reported functionalized semiconductor QDs (67) for the detection of NADH and their use to follow NAD$^+$-dependent biocatalyzed transformations (Fig. 33). 67 The reduction of the 67 capping layer by the NAD(P)H cofactors activated the fluorescence of the QDs, and provided a path for the optical detection of NADH. As a model system, the QDs (67) were applied to analyze ethanol in the presence of NAD$^+$-dependent alcohol dehydrogenase (AlcDH) (Fig. 33). In this system, AlcDH catalyzed the oxidation of ethanol to acetaldehyde with concomitant reduction of NAD$^+$ to NADH. The functionalized QDs were also introduced as optical labels into HeLa cancer cells and the intracellular metabolism and the effect of anticancer drugs on the cell metabolism were monitored.

The same group reported another phenyl boronic acid-functionalized CdSe/ZnS quantum dot (QDs 68) binding NADH or NAD$^+$ (Fig. 33). 68 The NAD$^+$-functionalized QDs were effectively quenched by an electron transfer process, while the NADH-modified QDs (68) were inefficiently quenched by the reduced cofactor. These properties enable the implementation of the QDs for the fluorescence analysis of ethanol in the presence of alcohol dehydrogenase.

**Fluorescent and colorimetric sensors for FAD**

Hong’s group developed a novel fluorescent chemosensor (65) that uses the Zn$^{2+}$–DPA complex, which selectively targets FAD from among other flavins in water (Fig. 32). 65 strongly bound to the diphosphate group of FAD and caused a change in the intramolecularly stacked conformation between adenine and the isoalloxazine ring in aqueous solution. The fluorescence intensity at 525 nm was increased ~6-fold upon addition of 2 equiv. of 65. It revealed that at pH 7.4, 65 could split FAD to form cyclic riboflavin 4,5-cyclic phosphate (cFMN) and adenosine 5-monophosphate (AMP).

The same group further explored the kinetic data for the splitting of FAD to cFMN, as catalyzed by 65 and another Zn$^{2+}$–DPA complex (66) (Fig. 32). 66 could efficiently split FAD to form cFMN and AMP with <1 mol% of a substrate,
The NADH-functionalized QDs were used for the optical analysis of 1,3,5-trinitrotriazine, an RDX explosive, with a detection limit of $1 \times 10^{-10}$ M.

A reversibly activatable T1 (relaxation)-weighted MRI/optical contrast agent (69), which was sensitive to NADH, was reported by Louie et al. (Fig. 34). In the dark, 69 in water favors the 69-MC (open) isomer which has strong fluorescence at 539 nm, with a quantum yield of 22%. In the presence of NADH, 69 underwent an isomerization to its 69-SO (closed) isomer. Removal of the phenoxide oxygen atom from the coordination sphere increased the hydration number, leading to a relaxivity increase of 54%, while the strong fluorescence was gradually quenched and disappeared in 20 minutes. After applying hydrogen peroxide to the system, the T1 value was fully restored but the fluorescence was only partially recovered. This finding revealed that both MC and SO isomers of 69 are non-toxic to cells and can be transported into cells.

Han’s group synthesized 70 to use as a chemosensor for NADH. 70 could recognize NADH by two mercury metal ions in the compound using the metal–anion interaction and its subsequent binding-induced fluorescence changes of FMA (Fig. 35). 70 showed a high selectivity for NADH over various anions and a high sensitivity of 1.2 μM for the detection of NADH. Additionally, this probe could discriminate between NADH and NAD$^+$ and could be used for the assay of enzymes related to the NADH/NAD$^+$ couple reaction.

**Concluding remarks**

In this review, we covered developments between 2004–2010 concerning development and use of exciting fluorescent and colorimetric probes for important biomolecules, such as XNP (where X = adenosine (A), uridine (U), cytidine (C), guanosine (G), and N = mono (M), di (D), tri(T)), FAD and NADH. We classified these various probes by their target biomolecule’s structural classifications. So far, most of the fluorescence and colorimetric detection studies have focused on the sensing of ATP. As a result, ATP chemosensors bearing imidazolium or quaternary ammonium groups as the binding motif, chemosensors based on hydrogen bond interactions, chemosensors using the zinc complex and the other metal complexes as the binding motif, and chemosensor-linked polymers or mesoporous materials have been reported. With fine tuning and careful designing, coupled with especially intelligent combinations of basic organic reactions and communicating methods, many successful probes for GTP, UTP, TTP, UDP, AMP, GMP, FAD and NADH have also been extensively studied. Additionally, the biological applications of these sensors were reviewed in the present paper. For example, fluorescence enhancement of 2 and 4 could be observed when stained HeLa cells were further incubated with ATP, indicating the possible usage in fluorescence images involving nucleoside polyphosphates within living cells; receptor molecule 33 for ATP can be used as a colorimetric staining agent for yeast (S. cerevisiae) cells and staining could be viewed by simple light microscopy; and ATP sensors 34 and 35 can be used for real-time fluorescence monitoring of enzyme reactions and in a fluorescence-imaging study of the nucleoside polyphosphates inside living cells, such as HEK293 and NIH3T3 cells. We believe this research area will become more active due to the significance of these biomolecules, and the applications resulting from further investigations will continue to increase.

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**Notes and references**