

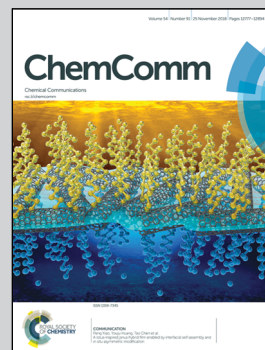


Showcasing research from Doctor Nao Xiao's group,
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Monitoring ADP and ATP *in vivo* using a fluorescent Ga(III)-probe
complex

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An *in situ* generated L- Ga^{3+} ensemble detected ADP and ATP
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Monitoring ADP and ATP *in vivo* using a fluorescent Ga(III)-probe complex†

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A naphthol-based sensor (L) was designed and synthesized for the specific recognition of Ga³⁺ using fluorescence enhancement. An *in situ* generated L–Ga³⁺ ensemble detected ADP and ATP more selectively through a fluorescence “switch off” response, which was confirmed both in cells and in adult zebrafish.

Gallium is a rare metal found in soil and is extensively used as a semiconductor material and in chemical synthesis, fuel storage and antitumor medication. However, Ga-based industrial wastes can contribute to environmental pollution, and especially, Ga-based harmful residues can further build up in the body leading to anemia and hypercalciuria, and inhibit osteoclast bone resorption.¹ Therefore, convenient visualization and quantitative detection methods are required for monitoring trace concentrations of gallium(III). A fluorescent sensor is a good candidate for Ga³⁺ detection. This type of method exhibits some advantages such as being convenient, noninvasiveness, carried out in real time, low price, requiring small sample amounts, and possessing high sensitivity and selectivity.^{2–10} However, just a few Schiff base-type fluorescent probes for the detection of gallium have been reported in recent years.^{1,11–17} Therefore, the development of new and improved chemosensors for the selective determination of Ga³⁺ is necessary.

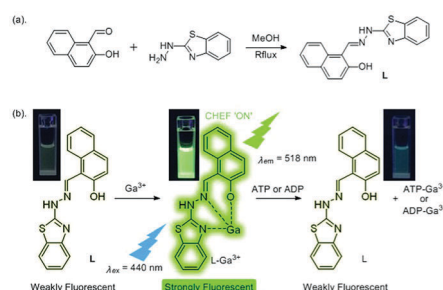
Adenosine 5'-monophosphate (AMP), adenosine 5'-diphosphate (ADP) and adenosine 5'-triphosphate (ATP) mainly function as energy substances in various biological processes. Deficiency in the adenosine-phosphate level is considered to be linked with many cellular processes. Monitoring of the adenosine-phosphate concentration level *in vitro* and *in vivo* is thus important for studying and understanding multiple cellular mechanisms. Therefore, some works have been devoted to

detect the structurally similar AMP, ADP or ATP.^{18–23} However, there are only a few reports that have been devoted to the detection of polyphosphates by Ga³⁺ complexes, although gallium possesses strong binding affinity for diphosphates and triphosphates.^{24–26}

In this work, we have designed and synthesized a fluorescent naphthol-based sensor **L** (Scheme 1a) containing electron-donating Schiff base N, thiazole N and phenolic OH, which provide binding sites to gallium ions. The naphthol ring appears to provide a rigid conjugated matrix. These features of **L** can specifically detect Ga³⁺ from other ions in dimethyl sulfoxide (DMSO) aqueous solution, accompanied by a strong green fluorescence enhancement. The L–Ga³⁺ self-assembled fluorescent sensor shows significant fluorescence turn-off only when ADP or ATP is present among anions. The possible application of L–Ga³⁺ as an *in vivo* or *in vitro* sensor of ADP or ATP is also reported by our fluorescence imaging results with living cells and adult zebrafish.

The chemosensor (**L**) was easily synthesized with a modified procedure using 2-hydroxy-1-naphthaldehyde and 2-hydrazinobenzothiazole with 89.20% yield (Scheme 1a).^{27,28} **L** was characterized by ¹H NMR, ¹³C NMR, and ESI-MS (Fig. S1–S3, ESI†). Further evidence was obtained from the crystal data of **L**, as shown in Fig. S36 and Tables S4–S6 (ESI†).

In order to investigate the effect of solvents on fluorescence enhancement when **L** is chelated with Ga³⁺, the fluorescence



Scheme 1 (a) Synthesis of naphthol-based chemosensor **L**. (b) The reversible sensing mechanism of **L** for Ga³⁺.

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responses of **L** were examined in dichloromethane (DMC), ethanol (EtOH), methanol (MeOH), acetonitrile (MeCN), dimethylformamide (DMF) and dimethyl sulfoxide (DMSO). The fluorescence response of **L** upon addition of Ga^{3+} in DCM, EtOH, MeOH, MeCN or DMF showed a relatively lower detection effect than that in DMSO, whereas **L** in all of the solvents exhibited weak fluorescence intensity in the absence of Ga^{3+} (Fig. S4, ESI†). Therefore, DMSO was selected as the solvent due to its largest fluorescence enhancement factor. An emission wavelength of 518 nm was employed to determine the most effective excitation wavelength, and 440 nm was observed to be the wavelength at which the highest fluorescence intensity was observed in DMSO (Fig. S5, ESI†). A change in the fluorescence intensity of L-Ga^{3+} was investigated with an increase in water content for DMSO solutions. The highest fluorescence intensity was observed with a 9/1 (v/v) DMSO/ H_2O (v/v) ratio (Fig. S6, ESI†). The addition of water to DMSO could quench the fluorescence intensity of L-Ga^{3+} . However, the fluorescence response of L-Ga^{3+} in absolute DMSO is weaker than that in $\text{DMSO/H}_2\text{O} = 9/1$ (v/v), which might be due to the addition of high polar solvents leading to improvement of the reaction. In the chromaticity diagram (CIE, 1931) (Fig. S7, ESI†), the chromaticity coordinates x and y of the fluorescence emission peak are 0.26 and 0.62, respectively, which illustrates that L-Ga^{3+} is a green fluorescent compound.

The UV-Vis absorption of **L** (5.0 μM) in 2.0 mL aqueous DMSO ($\text{DMSO/H}_2\text{O}$, v/v = 9/1) solution upon addition of Ga^{3+} ions was investigated. The absorption spectra of **L** changed upon addition of Ga^{3+} ions (Fig. S8, ESI†). The band at 370 nm noticeably decreased and two increased UV bands were observed at around 285 nm and 440 nm respectively. The presence of two clear isosbestic points at 305 nm and 415 nm was observed, indicating that a new product was formed from **L** upon binding with Ga^{3+} .^{15–17} The **L** solution exhibited a rapid color change from light yellow to green as the concentration of Ga^{3+} was increased from 0.0 μM to 50.0 μM , which could be observed by the naked eye. The responsiveness was determined using the time-response plot for binding sensor **L** with Ga^{3+} as given in Fig. S9 (ESI†). Following the addition of 10 equiv. Ga^{3+} ions to 5.0 μM sensor **L**, the UV-Vis absorbance at 440 nm gradually increased to a relatively stable value within 1 h. The appropriate response-time plot demonstrates that **L** possesses good responsiveness and adaptability to Ga^{3+} .

To validate the selectivity of **L**, UV-Vis response experiments of **L** (5.0 μM) in $\text{DMSO/H}_2\text{O}$ (v/v = 9/1) toward various metal ions, such as Li^+ , Na^+ , Mg^{2+} , Al^{3+} , K^+ , Ca^{2+} , Cr^{3+} , Mn^{2+} , Fe^{2+} , Fe^{3+} , Co^{2+} , Co^{3+} , Ni^{2+} , Cu^{2+} , Zn^{2+} , Ga^{3+} , Cd^{2+} , In^{3+} , Ba^{2+} and Ce^{2+} , were studied (Fig. S10, ESI†). The UV-Vis spectra and naked-eye appearance of receptor **L** were significantly perturbed by the addition of a number of metal ions in a more or less color changing fashion. Thus, the colorimetric detection of Ga^{3+} solution containing receptor **L** lacked selectivity. Since receptor **L** lacked the specificity towards any particular metal ion in colorimetric measurement as described above, the fluorescence emission response experiments of **L** toward various metal ions were further investigated to test its specificity towards a particular metal ion. As shown in the fluorescence spectra of Fig. 1, except Al^{3+} and In^{3+} which slightly increased the fluorescence

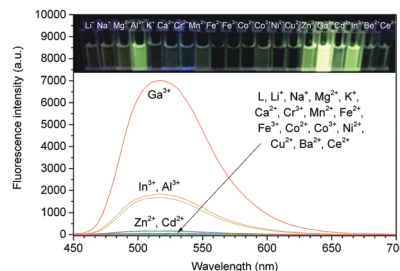


Fig. 1 The changes in fluorescence spectra of **L** (5.0 μM) in the presence of 10 equiv. of different metal ions in $\text{DMSO/H}_2\text{O}$ (v/v = 9/1) solution. $\lambda_{\text{ex}} = 440$ nm.

intensity, the addition of 10 equiv. of Ga^{3+} caused gradual enhancement of fluorescence until more than a 150-fold increase of the green emission band at $\lambda_{\text{max}} = 518$ nm (Fig. S11, ESI†). This indicated that the fluorescence intensity depended on the numbers of formed emitters of L-Ga^{3+} . The designed recognition with an off-on fluorescence change was achieved. Competition experiments were performed to explore the anti-interference ability of L-Ga^{3+} (5.0 μM , 1 : 10) by adding 10 equiv. of other metal ions. It was found that all competitive metal ions had no obvious interference to L-Ga^{3+} (Fig. S12, ESI†). This finding indicates that **L** can selectively distinguish Ga^{3+} from other metal ions.

We also studied the response of fluorescence lifetime and quantum yields (Φ) to the concentration of Ga^{3+} . As shown in Fig. 2, **L** initially showed a two-exponential decay, which limited the fluorescence emission. The average lifetime of $\tau_A = 4.16$ ns became longer, $\tau = 4.35$ ns, after addition of Ga^{3+} (Table S1, ESI†). When **L** chelated with Ga^{3+} , L-Ga^{3+} exhibited a single-exponential decay with almost the same lifetime. These results revealed that the fluorescence lifetime depends on the intrinsic photophysical properties of each emitter. As displayed in Table S2 (ESI†), the fluorescence quantum yields of **L** at $\lambda_{\text{em}} = 518$ nm increased from 35.46% to 60.93%, 89.20%, 85.90% and 82.71% in the presence of 0.5, 1.0, 2.0, 4.0 and 8.0 equiv. of Ga^{3+} , respectively. The fluorescence quantum yield of **L** measured in the presence of 2.0 equiv. Ga^{3+} was $\Phi = 0.892$, which was the highest value compared to those reported with other chemosensors. The slightly decreased values of $\Phi = 0.8590$ and $\Phi = 0.8271$ were the result of a self-absorption phenomenon.

The binding affinity of **L** towards Ga^{3+} was quantified based on fluorescence titration experiments using a Job plot, which

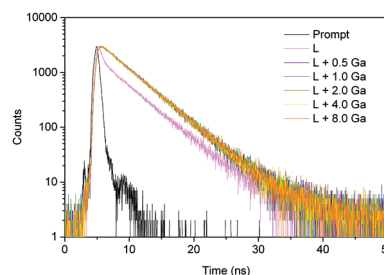


Fig. 2 The fluorescence decay curves (collected at 518 nm) of **L** itself and **L** with the addition of various equivalents (0.5, 1.0, 2.0, 4.0 and 8.0) of Ga^{3+} in $\text{DMSO/H}_2\text{O}$ (v/v = 9/1) solution.

showed that Ga^{3+} binds with **L** in a 1 : 1 stoichiometry (Fig. S13, ESI†). These fluorescence spectra implied a structural change in **L** upon complexation with Ga^{3+} . To clarify this structural change, ESI-MS analysis and ^1H NMR titration were carried out to further investigate the response mechanism. Upon addition of Ga^{3+} , the observed molecular-ion peaks appearing at 705.18, 705.07 and 706.99 were attributed to the isotopic peaks of $[\text{2L-2H} + \text{Ga}^{3+}]^+$ (calcd for $\text{C}_{36}\text{H}_{24}\text{GaN}_6\text{O}_2\text{S}_2$: 705.07, 706.07 and 707.06). These peaks almost disappeared with the formation of a new intense peak corresponding to $[\text{L-H} + \text{Ga}^{3+} + \text{DMSO}]^+$ (calcd for $\text{C}_{20}\text{H}_{19}\text{GaN}_3\text{O}_2\text{S}_2$: 466.02) (Fig. S14–S18, ESI†). The Job plot and ESI-MS results indicated a 1 : 1 binding model between **L** and Ga^{3+} in DMSO aqueous solution. The ^1H NMR spectra of **L** recorded in mixed DMSO- d_6 - D_2O solution upon increasing Ga^{3+} showed small but significant spectral changes which were due to the decrease in electron density of the **L** unit by Ga–N coordination (Fig. S19, ESI†). The phenolic OH of **L** did not appear in D_2O solution, indicating that the –OH protons were deprotonated. The proton signals at ~ 9.18 ppm and ~ 8.58 ppm were attributed to $\text{CH}=\text{N}$ and –NH– protons, respectively. The addition of Ga^{3+} gradually induced a downfield shift of the $\text{CH}=\text{N}$ proton from ~ 9.18 ppm to ~ 9.20 ppm, and finally the original signal was replaced by a new one. This finding indicates that the nitrogen atom of the $\text{CH}=\text{N}$ group was involved in the binding with Ga^{3+} . Chelation of **L** with Ga^{3+} led to a highfield shift of the –NH– proton from ~ 8.58 ppm to ~ 8.10 ppm, and the original signal was also replaced by a new one. This suggests that thiazolyl was involved in the binding with Ga^{3+} . Based on the ^1H NMR titration experiments and the structures of similar types of Ga^{3+} complexes reported earlier,^{13–15} **L** might bind with Ga^{3+} by –OH, =N– and –NH– in a tridentate fashion. Subsequently, the association constant (K_b) was determined to be about $5.54 \times 10^4 \text{ M}^{-1}$ using a Benesi–Hildebrand plot (Fig. S20, ESI†) by fitting the plots of fluorescence intensity against Ga^{3+} concentrations. Ga^{3+} could be detected down to 7.83 nM based on the $3\sigma/\text{slope}$ when 5.0 μM **L** was employed, which is the lowest value compared to those reported in the literature. The lowest detection limit of probe **L** with Ga^{3+} was determined from the linear relationship of fluorescence emission intensity at $\lambda_{\text{max}} = 518 \text{ nm}$, i.e., I_{518} vs. $[\text{Ga}^{3+}]$ having $R^2 = 0.9904$ upon titration of probe **L** with Ga^{3+} (Fig. S21, ESI†). **L** was compared with other Ga^{3+} fluorescent sensors, as presented in Table S3 (ESI†).

The mechanism of the fluorescence response of **L** to Ga^{3+} is the combination of ESIPT (excited state intra-molecular proton transfer) and CHEF (chelation enhanced fluorescence). Owing to $\text{C}=\text{N}$ and adjacent OH, **L** has the feature of ESIPT, which exhibits a weak emission at 518 nm as shown in Scheme 1b. The introduction of Ga^{3+} binding with $\text{C}=\text{N}$ and OH further restrains ESIPT. The chelation of **L** with Ga^{3+} led to the formation of a stable complex L-Ga^{3+} and, consequently, the chelated system enhanced fluorescence at 518 nm due to the CHEF.

Systemic studies confirmed that increasing the ratio of Ga^{3+} to **L** facilitated the absolute formation of L-Ga^{3+} , so a 1 : 2 ratio of **L** to Ga^{3+} was chosen for sensing anions in DMSO– H_2O (v/v = 9 : 1). The competitive experiments were carried out using L-Ga^{3+} (5.0 μM) with 10 equiv. of various anions, including I^- , F^- , Br^- ,

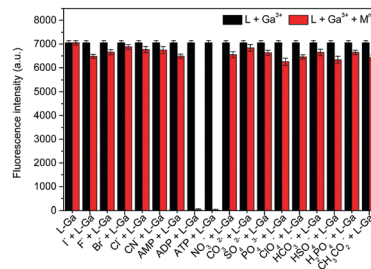


Fig. 3 Fluorescence responses of L-Ga^{3+} in the presence of various anions in DMSO/ H_2O (v/v = 9/1) solution. The bar of L-Ga^{3+} represents the addition of 10 equiv of Ga^{3+} to the solution of **L** (5.0 μM). $\lambda_{\text{ex}} = 440 \text{ nm}$. $\lambda_{\text{em}} = 518 \text{ nm}$.

Cl^- , CN^- , AMP, ADP, ATP, NO_3^- , CO_3^{2-} , SO_4^{2-} , PO_4^{3-} , ClO_4^- , HCO_3^- , HSO_4^- , H_2PO_4^- and CH_3CO_2^- in DMSO/ H_2O (v/v = 9/1) solution. Much to our delight, only very slight fluorescence changes at 518 nm with small error bars were observed when L-Ga^{3+} was exposed to these interferents, except ADP and ATP which quenched the fluorescence intensity (Fig. 3). Here, taking the assembled probe L-Ga^{3+} (the molar ratio is 1 : 2) with ATP as an example, the spectral changes were illustrated. As shown in Fig. S23 and S25 (ESI†), the emission intensity of L-Ga^{3+} at 518 nm constantly decreased until 5.5 equiv. of ATP were added. ATP greatly quenched the fluorescence of L-Ga^{3+} complexes, which suggested that ATP restrains **L** from interacting with Ga^{3+} and breaks the self-assembly of metal–ligand. The sensing ability of L-Ga^{3+} for ATP was further determined by fluorescence titration.

The major species with $m/z = 715.85$ was observed in mass spectra (Fig. S27, ESI†), which matched with the isotope patterns for $[\text{Ga}(\text{ATP})(\text{DMSO})(\text{MeOH})_2\text{-2H}]^+$ (calcd for $\text{C}_{14}\text{H}_{28}\text{GaN}_5\text{O}_{16}\text{P}_3\text{S}$: 715.97). The observation of $m/z = 320.43$ (calcd for $\text{C}_{18}\text{H}_{14}\text{N}_3\text{O}_5$: 320.09) pointed to the recovery of ligand **L** (Fig. S28, ESI†). Binding of ATP with Ga^{3+} was further confirmed by ^{31}P NMR spectral study (Fig. S30, ESI†). The clear differentiation of ^{31}P NMR signals was observed. In the presence of excess ATP, α -phosphorus atoms of ATP underwent upfield shifts, and the $\text{P-}\beta$ -atom experienced downfield shifts. Meanwhile, the broadening and shifting in ^{31}P NMR signals for α -, β -, and γ -P atoms confirm the binding to Ga^{3+} through the oxygen atom bearing the negative charge of the respective phosphate unit. These results confirmed that ATP recognition occurs from the extrusion of Ga^{3+} from the L-Ga^{3+} complex, accompanying the ESIPT from phenolic OH to the Schiff base N in **L**. The combination of L-Ga^{3+} and ATP resulted in the CHEF inhibition and ESIPT restoration with quenching of the bright green emission. It was assumed that ATP was attracted to Ga^{3+} through electrostatic interaction between positively charged Ga^{3+} and the phosphate group. The proposed mechanism is shown in Scheme 1b.

The effect of pH on **L** and L-Ga^{3+} was investigated. As shown in Fig. S31 (ESI†), an appropriate pH range for the detection of ADP or ATP is 6–8. MTT assays are shown in Fig. S34 (ESI†), indicating that more than 95% cells remain alive at the detected concentration of L-Ga^{3+} (1 : 2) (60 μM). Considering the potential application, the fluorescence properties of complex L-Ga^{3+} in culture medium (0.1% DMSO, v/v) solution were then studied

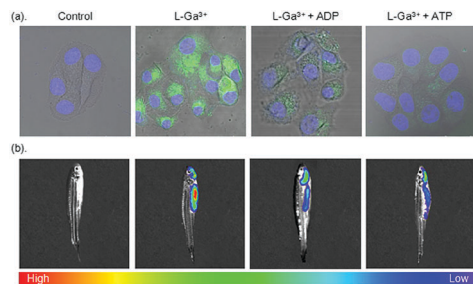


Fig. 4 (a) Confocal microscopy images of 16HBE cells subjected to ligation with L-Ga³⁺, followed by staining with Hoechst 33342 (blue, nucleus-specific dye) and ADP or ATP. (b) *In vivo* images of adult zebrafish; the fluorescence intensity recorded for the zebrafish incubated with L-Ga³⁺, with L-Ga³⁺, and then fed with ADP or ATP.

in detail. Compared to that in DMSO-H₂O (v/v = 9 : 1) solution, the fluorescence intensity of L-Ga³⁺ was quenched severely. But interestingly, L-Ga³⁺ caused a significant fluorescence enhancement in the culture medium, accompanied by the obvious hypochromatic shift of 18 nm to around 500 nm (Fig. S35, ESI[†]), which is probably due to a change in the polarity of the solvent systems. The enhanced fluorescence intensity was sequentially quenched by adding ATP. These results clearly indicated that L-Ga³⁺ can serve as a turn-off probe to detect ATP under physiological conditions. The L-Ga³⁺ complex was further used to map ATP in living cells by bioimaging. Normal human bronchial epithelial (16HBE) cells were first exposed to chemosensor L-Ga³⁺ (60 μM) for 1 h and then incubated with 120 μM of ATP for 1 h at 37 °C. Strong fluorescence was observed in the cells previously incubated with L-Ga³⁺. As shown in Fig. 4a, an apparent fluorescence signal appeared in the cytoplasm, but not in the nucleus. When the cells were first exposed to L-Ga³⁺, subsequently treated with ATP, the fluorescence signals from L-Ga³⁺ obviously decreased, indicating that the L-Ga³⁺ could provide a selective response to ATP levels in living cells. To assess the applicability of the proposed method, adult zebrafish were used to investigate whether L-Ga³⁺ can respond to ATP in complex environments. Strong fluorescence was noticed for the adult zebrafish incubated with L-Ga³⁺ (60 μM) for 1 h. The fluorescence signal was significantly quenched for the zebrafish pre-treated with exogenous ATP (120 μM) for 1 h before being exposed to L-Ga³⁺ (60 μM) for 1 h. The summarized results in Fig. 4b indicate the feasibility and reliability of the present method for ADP and ATP detection under practical biological conditions.

In summary, chemosensor **L** based on a naphthol fluorophore demonstrated a unique selectivity toward Ga³⁺ *via* fluorescence turn-on in DMSO aqueous solution. Furthermore, the L-Ga³⁺ ensemble demonstrated high selectivity and sensitivity toward ADP and ATP over anions in aqueous solution, which quenched the fluorescence of the ensemble by displacing the gallium ion from it. Spectral data analysis, including fluorescence and ESI-MS, suggested that the polyphosphate anions were likely bound to gallium during the sensing process. The fluorophore L-Ga³⁺ ensemble is capable of mapping ADP and ATP in cells and adult zebrafish. This finding shows

that L-Ga³⁺ offers potential value for ADP and ATP detection in clinical applications.

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Conflicts of interest

There are no conflicts to declare.

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