An ion-based chromogenic detecting method for phosphate-containing derivatives in physiological condition

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Abstract

The detection for phosphate-containing and ATP in water is a challenging task. Here, we report a simple and sensitive method for detecting both HPO$_4^{2-}$ and ATP in aqueous solution. The new ensemble is prepared by mixing ytterbium chloride and pyrocatechol violet in a 2:1 molar ratio in aqueous solution of 10 mM 2-(4-(2-hydroxyethyl)-1-piperazinyl)ethanesulfonic acid (HEPES) buffer at pH 7.0. Upon the addition of YbCl$_3$, the maximum absorption peak gradually shifted from 444 nm (yellow) to 623 nm (blue). With the addition of HPO$_4^{2-}$ or ATP solution into the system, the ensemble resulted in a change back of color from blue to yellow and caused a variation in UV–vis absorption spectra. The ensemble exhibits excellent selectivity for HPO$_4^{2-}$ and ATP over other common anions including Cl$^-$, SO$_4^{2-}$, CH$_3$COO$^-$, HClO$_4^-$ and HCO$_3^-$.

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1. Introduction

Phosphorus is one of the most important elements in lives. Together with heterocyclic bases and deoxyribose, phosphates make up the nucleotides, which in turn make up the genes, the hereditary elements of lives. More straight saying, nucleotide acting as frame unit of nucleic acid and participating in life activity processes of all cells is a very important biochemical substance in metabolizing. People have developed many technologies of detecting nucleotide such as spectrum analysis [1], mass spectrometry [2], chromatography [3] and electrophoretic method [4] in recent years. In addition, phosphate and its derivatives, particular adenosine triphosphates (ATP), play pivotal roles in energy utility and electrostatic interactions for the recognition of analytes [7,8]. These interactions are, however, hindered drastically in a highly polar medium such as water because of the competing solvation effect [9]. The detection for phosphate anions, and more important for ATP in water is, hence, a challenging task. Among those anion sensors [6] in aqueous media, significant progress has been made in the detection of oxy-anions (such as HCO$_3^-$, H$_2$PO$_4^-$) with lanthanide-containing receptors (especially Eu(III) and Tb(III)) by spectrometry and NMR methods [10,11]. During the investigation on biological effects of dinuclear lanthanide complexes stemmed from a phenol-based ligand, HB-bimp [12], (2,6-bis[benzimidazolylmethyl]laminomethyl)-4-methyl-phenol, we found that this system is an efficient sensor for HPO$_4^{2-}$ and ATP. It is prepared by mixing HB-bimp, YbCl$_3$, pyrocatechol violet (PV) in a 1:2:1 molar ratio...
in water solution. However, to our amazement and more
interesting, we discovered an even more simple and sensi-
tive sensor for both HPO$_4^{2-}$ and ATP [13]. The new en-
semble is obtained by mixing only YbCl$_3$ and PV in a 2:1
molar ratio in a 10 mM HEPES buffer aqueous solution at
pH 7.0.

2. Experimental

2.1. Materials

All the common chemicals were of analytical grade.
Pyrocatechol violet was purchased from Shanghai city of
China. Sodium monohydrogenphosphate was purchased
from Beijing city of China. Sodium pyrophosphate and
sodium hexametaphosphate were purchased from Tianjing city
of China. Adenosine 5$'$-monophosphate (AMP), ATP, ur-
dine 5$'$-diphosphate (UDP), uridine 5$'$-triphosphate (UTP)
and (-)-adenosine 3',5'-cyclic monophosphate 98% (cAMP)
were purchased from Aldrich. Guanosine 5$'$-triphosphate
tris–salt (ppGpp) was purchased from Sigma. Lanthanide ox-
ides were purchased from Rare Earth Graduate School of
China. HEPES was purchased from Sigma. Different pH HEPES
solutions were obtained by adding NaOH (0.1 M) and HCl (1 M)
using a Beckman/Phillips pH meter determining.

All kinds of lanthanide salts were prepared from lan-
thanide oxides and hydrochloric acid (37%). Lanthanide ions
solution was prepared by dissolving lanthanide chlorides into
to water.

2.2. Instrument

pH determinations were performed using a Beckman FS 50
pH meter. UV–vis spectra were recorded on a HP453 spect-
trophotometer. PO-120 quartz cuvette (10 mm) was pur-
chased from Shanghai city of China. $^1$H NMR spectra were
recorded on a Bruker DRX-400 MHz NMR spectrometer.

$^3$P$^1$H NMR spectra were recorded on a Bruker DRX-
400 MHz NMR spectrometer.

3. Results and discussion

3.1. UV–vis spectra

The new ensemble is prepared by simply mixing YbCl$_3$
and PV in a 2:1 molar ratio in an aqueous solution (10 mM
HEPES buffer [14], pH 7.0). Fig. 1a showed the UV–vis
spectra obtained when the solution of YbCl$_3$ was titrated
into the buffer of PV (50 $\mu$M) which has the maximum ab-
sorption peak at 444 nm exhibiting yellow. Upon the pro-
cessing of titration, the maximum absorption peak gradually
shifted from yellow ($\lambda_{\text{max}} = 444$ nm) to blue ($\lambda_{\text{max}} = 623$ nm).
Succeeedent addition of HPO$_4^{2-}$ or ATP solution to the above
mixture, the maximum absorption peak at 623 nm decreased
while the peak at 444 nm increased (Fig. 1b). Simultaneously
the ensemble’s color changed from blue to yellow. In Fig. 1a,
the maximum absorption peak at 444 nm (PV–HEPES) de-
creased in inversely proportional to quantity of Yb$^{3+}$, the
maximum absorption peak at 623 nm (PV–HEPES) steped
up with Yb$^{3+}$ increasing. Likewise, in Fig. 1b, when ATP
was added into the above system containing Yb$^{3+}$, the
maximum absorption peak at 623 nm (PV–HEPES–Yb$^{3+}$) de-
creased in directly proportional to [ATP]; on the other hand,
the maximum absorption peak at 444 nm (PV–HEPES–Yb$^{3+}$)
increased.

3.2. pH dependent

Recently, numerous chemosensors for phosphate ions
have been reported, but most of them use organic solvents
as the sensing medium [15,6]. Only a few display a positive
response towards phosphate ions in an aqueous environment
[16,17]. Well then, it is more significant to detect HPO$_4^{2-}$
and ATP in water at physiological pH. The effect of different
pH buffers of the system on detecting HPO$_4^{2-}$ and ATP was

![Fig. 1. (a) UV–vis spectra: HEPES 10 mM buffer, [PV] = 50 $\mu$M, YbCl$_3$ was added gradually, with $[\text{YbCl}_3]$ = 0–100 $\mu$M; (b) UV–vis spectra: with addition of $[\text{ATP}]$ = 0–100 $\mu$M to HEPES 10 mM buffer, $[\text{Yb}]/[\text{PV}]$ = 50 $\mu$M.](image-url)
also studied. We confected different pH solutions by adding NaOH (0.1 M) into HEPES (10 mM) and used a Beckman \Phi50 pH meter to determine. At pH < 6.5 or pH > 7.5, the ensemble did work well neither in changing of color nor in moving of UV–vis spectra. The UV–vis absorbance changes aforesaid all occurred in the range of 6.5–7.5, most distinctively at pH 7.0 (Fig. 2).

### 3.3. Selectivity over other anions

The measurement for ATP obeyed Beer-Lambert’s absorption law very well within the concentration range of 10–320 μM, with a $\varepsilon$ of 6993 (mol/L)$^{-1}$ cm$^{-1}$ [18]. Linear regression with the least square fitting gives a correlation coefficient to be 0.9992 (Fig. 1s). The ensemble exhibited

![UV–vis spectra of various pH values.](image)

**Fig. 2.** The UV–vis spectra of various pH values. Three lines represent the absorbance of PV (50 μM), the absorbance of [Yb$_2$(PV)$_2$] (50 μM PV, 100 μM YbCl$_3$), and the absorbance of the ensemble when 100 μM ATP was added into the solution of [Yb$_2$(PV)$_2$].
excellent selectivity towards HPO$_4^{2-}$ and ATP over other common anions, including Cl$^-$, SO$_4^{2-}$, CH$_3$COO$^-$, HCO$_3^-$ and ClO$_4^-$ and so on as shown in Fig. 3, although the concentration ratios of HPO$_4^{2-}$, ATP and other common anions get to 1:1000. The selectivity of the sensor towards anions was more single than one reported by Hong et al. [19]. Compared with those synthetic receptor and designed peptide receptor by waters [20], the system showed strong recognition to phosphate ions and ATP in an aqueous solution. Not only adding other anions to PV–HEPES–Yb$^{3+}$ system could not bring about changes of the system in color and UV–vis but also their existence (1000:1) did not interfere with detecting HPO$_4^{2-}$ and ATP (Fig. 4). To our knowledge, the numbers of efficient analytical methods for phosphate ions with good selectivity over other common anions in aqueous solution are much less than that in organic solvents [21].

3.4. Stoichiometric relation and stability constants

What is the stoichiometric relation about our system? To answer this, we carried out experiments of isothermal spectrophotometric titration (IST) and plotted the curve of [Yb$^{3+}$] against $\Delta A$, the absorbance differences of the ensemble before and after the addition of Yb$^{3+}$, and (ATP) versus $\Delta A$, [HPO$_4^{2-}$] versus $\Delta A$ as well (Fig. 5). From the data, the coordination ratio of Yb to PV was found to be 2:1, while for ATP, Yb:ATP was consistent to be 1:1 which was also suitable to Yb:HPO$_4^{2-}$. Furthermore, we also calculated the conditional binding constants and thermodynamic parameters of the complexes related to the following equations:

$$2\text{ATP} + \text{Yb}_2(\text{PV}) = \text{PV} + 2\text{YbATP} \quad K_{\text{Yb2-PV}}^{298\text{K}} = 1.5 \times 10^{14}$$

$$2\text{ATP} + 2\text{Yb}^{3+} = 2\text{Yb(ATP)} \quad K_{\text{Yb-ATP}}^{298\text{K}} = 5.85 \times 10^{15}$$

$$2\text{HPO}_4^{2-} + \text{Yb}_2(\text{PV}) = \text{PV} + 2\text{YbHPO}_4^{2-} \quad K_{\text{Yb2-PV}}^{298\text{K}} = 5.07 \times 10^1$$

$$2\text{HPO}_4^{2-} + 2\text{Yb}^{3+} = 2\text{Yb(HPO}_4^{2-}) \quad K_{\text{Yb-HPO}_4}^{298\text{K}} = 2.0 \times 10^{15}$$

Table 1 summarized the thermodynamic parameters and stability constants for the metal complexes derived from PV and analytes by IST. From the data, one could find that HPO$_4^{2-}$ and ATP bound with Yb$^{3+}$ much more tight than with PV, quantitatively, over 4 orders. We thought that the coordination reactions were driven both enthalpically and entropically. It is well known that phosphate ions exist mostly with divalent anion hydrogenphosphate and univalent anion.

<table>
<thead>
<tr>
<th>PV</th>
<th>ATP</th>
<th>HPO$_4^{2-}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta H$ (kJ mol$^{-1}$)</td>
<td>$-40.29$</td>
<td>$-35.37$</td>
</tr>
<tr>
<td>$\Delta G$ (kJ mol$^{-1}$)</td>
<td>$-66.12$</td>
<td>$-89.94$</td>
</tr>
<tr>
<td>$\Delta S$ (J K$^{-1}$ mol$^{-1}$)</td>
<td>$86.67$</td>
<td>$183.1$</td>
</tr>
</tbody>
</table>

Table 1 Thermodynamic parameters and stability constants ($K$) for the binding of PV, ATP and HPO$_4^{2-}$ to Yb$^{3+}$ in an aqueous solution.
Fig. 5. (a) In a PV (50 μM) solution, the value of ΔA changes with the addition of Yb3+ (final concentration is 0–200 μM); (b) in the solution of Yb2 (PV) (50 μM), the value of ΔA changes with the addition of [ATP] (final concentration is 0–200 μM; (c) in the Yb2 (PV) (50 μM) solution, the value of ΔA changes with the addition of HPO42− (final concentration is 0–200 μM).

Fig. 6. Line (I) indicates the absorbance value changes at 444 nm UV–vis spectrum, line (II) indicates the absorbance value changes at 623 nm UV–vis spectrum.

3.5. The reversibility of the anions recognition

As a sensor, it implies that the measurement process is a reversible cycle. Adding secondly Yb3+ into the system of having detected ATP, it was found that the solution having changed yellow renewed blue and the maximum absorption peak (λmax = 444 nm) shifted to 623 nm. When we again added ATP into the above blue solution, the ensemble returned to yellow from the foregoing blue and caused obvious change of UV–vis absorption spectra, namely, the absorption peak at 623 nm decreased while the peak at 444 nm increased to come back to the respective maximum values (Fig. 6). The curves imply that repeatedly adding Yb3+ and ATP into PV (50 μM) containing HEPES buffer solution, UV–vis spectrum and color of system could change regularly. This fact could prove the reversibility of anion recognition.

3.6. Other phosphate-containing derivatives

We have mentioned that the new sensor exhibited excellent selectivity towards HPO42− and ATP over other common anions. But how about was its selectivity relative to GTP, CTP, TTP, UTP, AMP, ADP and pyrophosphate anion? One would speculate that all NTPs and pyrophosphate anion would bring about the similar color variety with the sensor. This was indeed the case. One fact was sure, that is, cAMP, AMP and ppGpp were totally incapable of causing the color change, even if its quantity is 10 times as much as ATP’s (Fig. 2a). As for ADP, it was able to give rise to the color change gradually with double quantity of ATP and resulted in a final color of yellow-green [22]. We also calculated the stability constant KYb–UDP 298 K = 1.39 × 1013 (Fig. 3a), which was 2 orders lower than constant of ATP. Simultaneously, it was proved that the sensor was more sensitive to ATP.

3.7. Possible mechanism

In our experiment, the color variation was very distinct. What was the possible structure of species that organized by dihydrogenphosphate, hardly without trivalent anion phosphate at the pH of the experiments. Moreover, in our experiments, the coordination ratio of Yb3+ binding with phosphate is foreign to phosphate’s valence. So we only calculated the binding constant of Yb and HPO42−.
Yb$^{3+}$ ions with the ligands? It is known that the yellow color of PV (the indicator) at neutral pH may change to blue when the molecules bind to metal ions [23]. In water solution, the molecule of PV would occur at acid/base equilibrium (Fig. 7).

Form I would initiate a complexation reaction with metal ions and give the deprotonized form II with two catechol groups in favor to form complex when encounter another metal ion. Why did we conclude the scheme of color changes in the text? We got the spectra of some acidity (pH = 0.01, 3.12, 7.0 and 10.34) solutions. The Fig. 4 s showed that the maximum absorption peak of PV would change gradually from shorter wave (444 nm about) to longer wave (600 nm about) with the increasing acidity. Under weaker acidity, PV exists mainly in form I; with the increase of acidity, it will change from I to II. Here, Yb$^{3+}$ can play the role of acid. That is, at first, Yb$^{3+}$ coordinates with I at position (1) and (2), this will promote the formation of II and its complexation. Accordingly, the maximum absorption peak of pyrocatechol violet will change from 444 nm to 623 nm. Moreover, it is the most perfect in a 1:2 molar ratio for PV-Yb$^{3+}$ in our experiment, so we deduce that Yb$^{3+}$ also coordinate in (3) and (4) position.

As a strong Lewis acid, Ln$^{3+}$ ions prefer O atoms as their coordination sites and the most common coordination numbers are 8 or 9, with favored structures [24,25]. Since it was found by the experiment that the best performance occurred when a 1:2 molar ratio of PV-Yb$^{3+}$ was adapted, we proposed a coordinating form on the left of Fig. 8 when Yb$^{3+}$ ions coordinate with PV. Once adding ATP

![Fig. 7. Acid/base equilibrium of PV.](image)

![Fig. 8. The proposed structure organized by Yb$^{3+}$ ion when it coordinates with PV (left) and with ATP (right) to form β,γ-Yb–ATP or α,β,γ-Yb–ATP complexes.](image)
into the system, competition between ATP and PV for Yb$^{3+}$ will take place. It is most possible that PV was released from the complex when ATP coordinates to Yb$^{3+}$, forming a new coordinating form. What was the new coordinating form? It is known that ATP can form two kind of complexes with Mg$^{2+}$, i.e. $\beta$-$\gamma$-Mg-ATP and $\alpha$-$\beta$-$\gamma$-Mg-ATP [5], and Yb and Mg are of similar size and have similar coordination requirements. So we could deduce the possible coordinating form (Fig. 8, on the right).

The extrication of PV should take the responsibility for the color changing back to yellow. Why is the sensitivity of the sensor to different phosphate-containing derivatives being phosphate ions $\approx$ ATP $>$ UDP $\gg$ AMP? We know Yb$^{3+}$ and PO$_4^{3-}$ are a hard acid and base, respectively. Yb$^{3+}$ is easy to bind with PO$_4^{3-}$ according to the rule of acid and base combining (hard acid affiniting hard base). We also gave the possible coordination form of Yb-HPO$_4^{2-}$ (Fig. 5s). In ATP molecule, the sugar ring is a soft base which interrates PO$_4^{3-}$ adjacent to it and softening intensity order is $\alpha$-PO$_4^{3-} > \beta$-PO$_4^{3-} > \gamma$-PO$_4^{3-}$. Among phosphate-containing derivatives such as AMP, UDP and ATP, ATP molecule contains a $\gamma$-PO$_4^{3-}$ which hardly be affected by the sugar ring; $\beta$-PO$_4^{3-}$ of UDP is soften by sugar ring to a certain extent. This was consistent to the above stability constants $K_{Yb-ATP}^{298 K} = 5.85 \times 10^{15} > K_{Yb-UDP}^{298 K} = 1.39 \times 10^{13}$.

While AMP has only an $\alpha$-PO$_4^{3-}$ which is softened to a great extent, this makes the $\alpha$-PO$_4^{3-}$ not easily coordinate with Yb$^{3+}$.

In order to confirm that HEPES did not take part in coordinating, $^1$H and $^{31}$P($^1$H) NMR spectra were recorded (Fig. 9); Yb$^{3+}$ did not affect $^1$H NMR spectrum of HEPES, however, $^1$H NMR spectra of PV and ATP took place big changes.

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**Fig. 9.** (a) $^1$H NMR spectra of HEPES and HEPES–Yb; (b) $^1$H NMR spectra of PV and PV–Yb; (c) $^1$H NMR spectra of ATP and ATP–Yb; (d) $^{31}$P($^1$H) NMR spectra of ATP and ATP–Yb.
because of adding Yb\(^{3+}\) into their \(\text{D}_2\text{O}\) solution. From these facts, the possibility of HEPES participating in coordination was excluded. Also \(^{31}\text{P}\{^1\text{H}\}\) NMR spectrum of ATP indicated that the signals of ATP in ATP were greatly affected by Yb\(^{3+}\) and shifted markedly to downfield.

### 3.8. Other metal ions

The detection course can even be observed with naked eyes (Fig. 10). Sensors that can detect analytes by the naked eye, without resorting to any spectrometer, is of particular interest because of its convenience. Only a few such sensors have, however, been reported [26]. Here, it should be pointed out that Yb\(^{3+}\) is the best candidate among the metal ions in our simpler ensemble. Other lanthanide ions are incapable to work as well as Yb\(^{3+}\) does, although they can play the similar part. Specifically, we find that not only the water solubility of corresponding salts of La\(^{3+}\), Nd\(^{3+}\), Sm\(^{3+}\) and Pr\(^{3+}\) is bad but also the UV–vis spectrum did not change distinctly and regularly (Fig. 6) when La\(^{3+}\), Nd\(^{3+}\), Sm\(^{3+}\) or Pr\(^{3+}\) were added into the detection ensemble. As for ions as Gd\(^{3+}\), Er\(^{3+}\) and Ho\(^{3+}\), though the UV–vis spectra gave rise to some variations (Fig. 6a), precipitations were produced quickly. Ce\(^{3+}\) did not work at all. It is a pity that Eu\(^{3+}\) and Tb\(^{3+}\) do not form stable complexes in water solutions. As for the transitional metal ions, we chose a few kind of typical metal ions as Ni\(^{2+}\), Mn\(^{2+}\), Fe\(^{3+}\), Zn\(^{2+}\) and Cu\(^{2+}\) to test. They could not do the same job (Fig. 6c). Particularly speaking, when Zn\(^{2+}\) was added into HEPES buffer containing PV, the ensemble changed from yellow to yellow-black and UV–vis apparently take place regularly changes (Fig. 6d). Adding Cu\(^{2+}\) into PV–HEPES system, the color of solution changed from yellow to blue and UV–vis apparently take place regularly changes (Fig. 6e). However, the sensor was unable to detect ATP because the ensemble had not any response to adding ATP. As for Fe\(^{2+}\), it could make PV–HEPES system change into yellow-green but did not bring the UV–vis changes (Fig. 6f). As regards Ce\(^{3+}\), Ni\(^{2+}\), Mg\(^{2+}\) and Mn\(^{2+}\) did not work at PV–HEPES system at all.

### 4. Conclusion

In summary, we have developed a simple and efficient colorimetric measuring method for phosphate anions and ATP by simply mixing YbCl\(_3\) and PV, a commercially available dye, in water at neutral pH. The reversibility of the anion recognition is demonstrated in Fig. 6. Yb\(^{3+}\) as a sensor can detect phosphate anions and ATP both spectrophotometrically and visually, with high selectivity over a variety of mono- and divalent anions. This sensor also allows a quantitative assay of phosphate-containing derivatives in aqueous solution down to the concentration range around \(10^{-5}\) M which is more sensitive than \(10^{-4}\) M presented by Miranda [27]. Compared with the method of Paker [10,11], our sensor is simpler and selective in recognizing phosphate-containing derivatives in aqueous solution. Now that ATP exists abroad in cell nucleus, cytoplasm and mitochondria and plays a bridge role in the energy metabolizing of cell. We can analyze the metabolizing process of cell by detecting the contents of ATP. The new ensemble should be hopefully acquired some applications in biological and medicinal sciences. A practical development to analyze urine sample is being carried out in our lab.

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### Appendix A. Supplementary data


### References


[18] See supporting information Table 1 s and Fig. 1 s.


[22] See Fig. 2 s in supporting information.


Biographies

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