Carbon dots as fluorescent probes for detection of VB$_{12}$ based on the inner filter effect†

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In this study, we constructed a new fluorescent sensing for VB$_{12}$ and investigated the mechanism of vitamin B$_{12}$ (VB$_{12}$) quenching fluorescence of carbon dots (CDs). The fluorescence suppression is attributed to the inner filter effect (IFE) because of the overlap between UV-vis absorption spectrum of VB$_{12}$ and emission/excitation spectra of CDs. This CDs-based sensor provides obvious advantages of simplicity, convenience, rapid response, high selectivity and sensitivity, which has potential application for the detection of VB$_{12}$ in the medical and food industry.

Introduction

Vitamin B$_{12}$ (VB$_{12}$), also called cobalamin, is an essential water soluble nutrient that can be found in foods such as meat, eggs and dairy products. It can effectively prevent diseases such as pernicious anemia, senile dementia, depression. Therefore, VB$_{12}$ plays an integral role in keeping the human body healthy. However, excess VB$_{12}$ can also produce toxic side effects, such as a lack of folic acid. So, the detection of VB$_{12}$ is very important.

In the past years, numerous analytical methods for the determination of VB$_{12}$ in different sample matrices have been successfully developed, including microorganism method, HPLC-UV, atomic absorption spectroscopy, thin layer chromatography, etc. However, most of these methods are not perfect due to the requirement of expensive equipment, being time consuming or complicated sample pretreatment. Therefore, a simple, sensitive, and selective method for VB$_{12}$ detection is highly demanded. Fluorescence analysis has been applied in the detection of VB$_{12}$ due to its high sensitivity and easy operation. In 2014, Kolekar’s group$^1$ detected VB$_{12}$ based on FRET between Cds and VB$_{12}$, and this method could be used in serum and urine actual samples. Other groups used rhodamine B$^2$ or gold nanoclusters modified by bovine serum protein$^3$ as fluorescent probes to detect VB$_{12}$. However, these methods had the following problems: the fluorescent probes had biological toxicity, the operations were complex and the sensitivities were not high. So choosing a green fluorescent probe and a simple operation can help constructing a better fluorescent method to detect VB$_{12}$.

Compared with traditional quantum dots and fluorescent dyes, carbon dots (CDs) have a series of unique properties such as excellent water solubility, anti-photobleaching, easy of modification, low toxicity, low cost, tunable excitation and emission spectra.$^{4-7}$ These attractive features indicate the prominent advantages of CDs in chemical sensing, biosensing, bioimaging, nanomedicine and catalyst. CDs have been applied in the sensing of ions (such as Hg$^{2+}$, Cu$^{2+}$, Fe$^{3+}$, F$^-$ and BrO$_3^-$, etc.),$^8-12$ molecular substances (for instance, glucose,$^{13}$ hemoglobin,$^{14}$ p-nitrophenol$^{15}$ etc.) and pH.$^{16}$ The mechanisms of CDs-based fluorescence sensor include photo induced electron transfer (PET), intramolecular charge transfer (ICT), fluorescence resonance energy transfer (FRET) and twisted intramolecular charge transfer (TICT), etc. Inner filter effect (IFE) is also a mechanism of fluorescence sensing. The IFE refers that the excitation and emission spectra of fluorophores are absorbed by absorbers, then the fluorophores suppress.$^{17}$ In the beginning, IFE was an inevitable error, the research on the IFE mainly focused on the correction of IFE. Since metal nanoparticles have good absorption coefficient, they can be used as good absorbers.$^{18}$ Now, the IFE has been developed into an analytical method.$^{19-24}$ Fluorescence detection based on IFE includes two forms. IFE can happen between metal nanoparticles and other fluorophores, and an analyte can make fluorescence recovery. IFE can also happen between an analyte and fluorophores, and the analyte can be detected based on fluorescence quenching. In this study, VB$_{12}$ was detected based on the IFE between VB$_{12}$ and CDs. This method has an extremely low LOD for detecting VB$_{12}$ of 93 nM, and the detection system is simple and fast.

Experimental

Reagents and equipments

Ammonium citrate (AR) and all the coexisting vitamins were obtained from Aladdin Chemistry Company Limited (Shanghai, China). Vitamin B$_{12}$ (VB$_{12}$) was purchased from Beijing Fangcao Medicine Chemical Industry Developed Company.

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NaH₂PO₄·2H₂O, Na₂HPO₄·12H₂O, NaOH, H₂PO₄, H₃BO₃, CH₃COOH and all the coexisting ions were obtained from Guoyao Company (Shanghai, China). VB₁₂ tablets were purchased from Yunpeng Medical Company (Shanxi, China) and VB₁₂ injections were obtained from Ruicheng Tiantong Company Limited (Shanxi, China). All chemicals were used in the experiments without further purification. Deionized water, purified by Millipore system (18.0 MΩ cm at 25 °C), were employed for all experiments.

pH was measured by a Model 1828 digital pH meter. PL spectra were acquired by a Hitachi F-7000 spectrometer. UV-vis absorption spectra were recorded by a Shimadzu UV-2600 spectrophotometer. FTIR-4800S spectra were employed for obtaining IR spectra in KBr discs in the 4000–400 cm⁻¹ region. X-Ray Diffraction (XRD) results were recorded on a Rigaku Smart lab with a speed of 6° per minute. Transmission electron microscopy (TEM) experiments were done on a Tecnai-F30 system. Zeta potential and dynamic light scattering (DLS) size distribution were obtained by a Zetasizer Nano.

Preparation of CDs

The fluorescent CDs were prepared through a simple and low-cost hydrothermal treatment.²⁹ Briefly, 2.0 g of ammonium citrate was added into 25 mL distilled water. The solution was heated from room temperature to 160 °C in a 50 mL para polyphenol (PPL) equipped stainless steel autoclave and held at 160 °C for 6 h. The color of the solution gradually turned into deep blue from colorless in appearance. When the resulting solution was cooled to room temperature, the solution was placed in the fridge for further application. The product can be used directly without any further passivation or purification.

Vitamin B₁₂ Sensing

For sensing vitamin B₁₂ (VB₁₂), 1.2 μM CDs solution and 2 mL 0.2 M PB buffer solution of pH 7.0 were mixed with the solution containing VB₁₂ of various concentration to afford a fixed volume of 5 mL. After stirring, the mixed solution was maintained at room temperature for 15 min, PL spectra were measured. Moreover, the experiments of coexistence of vitamins or heavy metal ions were conducted for further investigating the selectivity under similar experimental conditions of this sensing system. The excitation wavelength was set as 350 nm.

Interference experiments of VB₁₂

To investigate the effects of other coexisting substances to VB₁₂ detection, some vitamins (VB₁, VB₃, VB₅, VB₇, VB₉, VC) and some metal ions (Ba²⁺, Ca²⁺, Cd²⁺, Co²⁺, Cr³⁺, Cu²⁺, Zn²⁺, Ni²⁺, Mn²⁺, Fe²⁺, Fe³⁺, Hg²⁺, Pb²⁺) were added to CDs solution with VB₁₂. The concentration of Hg²⁺ was 10 μM, Fe³⁺ and Fe²⁺ were 40 μM. Then vitamins and other metal ions were 100 μM.

Results and discussion

Synthesis and characterization of CDs

The CDs with blue fluorescent were prepared by a hydrothermal treatment. The optical properties of the CDs are shown in Fig. S1.† As shown in the UV-vis absorption spectra, one absorption peak was at 234 nm, due to π → π* transition of C=O bond,²⁹ and another peak at 338 nm was attributed to n → π* transition of C=O bond.²⁹ The photoluminescence (PL) spectra of CDs showed that the maximum peak centered at 440 nm under a 350 nm excitation wavelength, which presented a bright blue color under UV lamp. And the emission spectra didn’t shift red with the excitation changing.

The QY of CDs was 0.24 by calibrating against reference quinine sulfate in H₂SO₄ and the lifetime was 6.52 ns fitting with single index. From Fig. 1, we found that the as-prepared CDs had an average size of 3.03 nm and they were monodispersed. XRD showed the CDs had 002 facet which means graphene layers. The FTIR spectrum showed the surface of CDs exist amide functional, hydroxyl groups and carboxyl groups. Zeta potential results showed a value of −11.73 mV, probably resulted from the slight ionization of hydroxyl group of the surface of the resulting CDs. DLS measurement revealed that the CDs particles had good size distribution with an average size of 9.68 nm. The synthesized CDs had good light stability, as showed in Fig. S2.†

The optimization of important factors for the probe

The effects of pH on the PL of CDs were investigated. As shown in Fig. 2(a), under acidic pH, the PL intensity of CDs decreased. This could be attributed to the carboxyl groups on the CDs surface accumulated by combining the protons. The PL intensity and the quenching kept almost constant in the pH range of 6.0–8.0. In this experiment, we selected pH = 7.0. Types of buffer were also investigation. In PB buffer, the most quenching was obtained as shown in Fig. 2(b). Then the reaction was fully
could be calculated to be 93 nM, according to the equation of LOD
tained to be 0.9940. The limit of detection (LOD) was also
increasing VB12 concentrations (0, 0.3, 0.5, 2.0, 4.0, 6.0, 8.0,
found that the PL intensity of CDs gradually decreased with
environment and less pollution. The detection system is simple
completed within 15 min, as shown in Fig. 2(c), suggesting
a rapid method to detect VB12. The CDs concentration was
established within 15 min, as shown in Fig. 2(c), suggesting
a rapid method to detect VB12. The CDs concentration was
investigated and the optimal concentration was 1.2 μM.

Establishment of the sensing system for VB12

Fig. 3(a) shows the PL response of the CDs towards VB12. It was
found that the PL intensity of CDs gradually decreased with
increasing VB12 concentrations (0, 0.3, 0.5, 2.0, 4.0, 6.0, 8.0,
10.0, 12.0, 15.0 μM). Fig. 3(b) shows a linear correlation curve
could be fitted between VB12 concentration and (F0 – F)/F0. In
which F0 is the PL intensity of CDs without VB12 and F with
different VB12 concentrations. A good linear range was within
0.3–15 μM and the linear correlation coefficient could be ob-
tained to be 0.9940. The limit of detection (LOD) was also
calculated to be 93 nM, according to the equation of LOD = 3σ/
k, in which σ is the standard deviation from 11 blank solutions
and k is the linear slope fitted. Compared with other methods of
fluorescence detection of vitamin B12, this method is green
environment and less pollution. The detection system is simple
and fast, the linear range is wide and the sensitivity is high, as
shown in Table 1.

Table 1 Comparison of our proposed method and the reported cases
to detect VB12 based on PL

<table>
<thead>
<tr>
<th>Sensor</th>
<th>Linear range (μM)</th>
<th>LOD (nM)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Graphene oxide nanolayer</td>
<td>0–1.08</td>
<td>320</td>
<td>1    (ref. 18)</td>
</tr>
<tr>
<td>CdS</td>
<td>3.7–73.8</td>
<td>5.1</td>
<td>2    (ref. 19)</td>
</tr>
<tr>
<td>CdTe</td>
<td>0.7–1.8</td>
<td>0.1</td>
<td>3    (ref. 20)</td>
</tr>
<tr>
<td>CDs</td>
<td>0.3–15</td>
<td>93</td>
<td>This work</td>
</tr>
</tbody>
</table>

Effect of coexisting substances in the determination of VB12

In order to explore the possibility of practical application in the
determination of VB12, the interferences from other vitamins
and anions were tested under the optimized conditions. Fig. 4
showed other vitamins have no influence to the detection of
VB12. And Table 2 showed that some anions quenched the
fluorescence of CDs, such as Hg2++. From Fig. S3† the coexisting
Hg2++ could suppress the PL intensity of CDs heavily while the
effect of Hg2++ coexisting could be eliminated by adding EDTA.

Fluorescence quenching mechanism of CDs by VB12

It was found that the fluorescence of CDs could be quenched by
VB12, revealing the possibility of applying the as-prepared CDs
as a sensitive fluorescent sensor of VB12. From the spectra in
Fig. 5, we could see a good spectral overlap between the
absorption spectrum of VB12 and the excitation and emission
spectra of CDs, suggesting that the fluorescence quenching
might be related to FRET or IFE. Since lifetimes of CDs both in
the absence and presence of VB12 remained unchanged (Fig. 6),
fluorescence quenching was unreasonable ascribed to the FRET
process. Then there wasn’t blue shift or red shift of CDs emis-
sion which shows no interaction appeared between CDs and
VB12. Furthermore, the selectivity toward VB12 could be
explained by the IFE mechanism [Fig. 7]. The IFE can be esti-
mated according to the following equation,25

\[
\frac{F_{corr}}{F_{obsd}} = \frac{2.33A_{ex}}{1 - 10^{-\frac{dA}{ex}}} \cdot \frac{A_{em}}{1 - 10^{-\frac{dA}{em}}}
\]

(1)

wherein, F_{obsd} is the measured maximum fluorescence intensity
and F_{corr} is the corrected maximum fluorescence intensity by
removing IFE from $F_{\text{obsd}}$. $A_{\text{ex}}$ and $A_{\text{em}}$ represent the absorbance at the excitation wavelength ($\lambda_{\text{ex}} = 350 \text{ nm}$) and maximum emission wavelength ($\lambda_{\text{em}} = 446 \text{ nm}$), respectively; $s$ is the thickness of excitation beam (0.10 cm$^2$), $g$ is the distance between the edge of the excitation beam and the edge of the cuvette (0.40 cm) and $d$ is the width of the cuvette (1.00 cm). In Table S1,† CF (correction factor) was that $F_{\text{cor}}/F_{\text{obsd}}$, which could be calculated by eqn (1.1). In order to ensure the credibility of the correction, the maximum value of CF could not exceed 3. $F_{\text{cor}}$ was the PL intensity of CDs with different VB$_{12}$ concentrations after IFE correction and it could be calculated by eqn (1).

The correction factor at each concentration of VB$_{12}$ thus could be calculated (Table S1†), $F_{\text{cor,o}}/F_{\text{cor}}$ was the corrected fluorescence intensity in the absence of VB$_{12}$. After removing the IFE, the suppressed efficiency $E = 1 - F/F_{0}$ for the totally observed and the corrected fluorescence of VB$_{12}$ was figured out, as shown in Fig. 8. We found that approximately all of the quench effects come from the IFE of VB$_{12}$.

**Practical application**

The method in real application was also investigated in the determination of VB$_{12}$ in injections and tablets samples. VB$_{12}$ samples of various known concentrations were employed to conduct the recovery experiments. The experimental results were summarized in Table 3. The recoveries ranging from

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### Table 2 Effect of metal ions (8 μM) on the detection of VB$_{12}$

<table>
<thead>
<tr>
<th>Coexisting substance</th>
<th>Concentration (μmol L$^{-1}$)</th>
<th>Change of fluorescence intensity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ba$^{2+}$</td>
<td>100</td>
<td>+0.01</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>100</td>
<td>+3.50</td>
</tr>
<tr>
<td>Cd$^{2+}$</td>
<td>100</td>
<td>+3.07</td>
</tr>
<tr>
<td>Co$^{2+}$</td>
<td>100</td>
<td>+5.13</td>
</tr>
<tr>
<td>Cr$^{3+}$</td>
<td>100</td>
<td>+2.42</td>
</tr>
<tr>
<td>Pb$^{2+}$</td>
<td>100</td>
<td>−5.31</td>
</tr>
<tr>
<td>Cu$^{2+}$</td>
<td>100</td>
<td>+0.77</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>100</td>
<td>+0.80</td>
</tr>
<tr>
<td>Ni$^{2+}$</td>
<td>100</td>
<td>+2.09</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>100</td>
<td>+3.60</td>
</tr>
<tr>
<td>Fe$^{2+}$</td>
<td>40</td>
<td>+2.81</td>
</tr>
<tr>
<td>Fe$^{3+}$</td>
<td>40</td>
<td>+2.87</td>
</tr>
<tr>
<td>Hg$^{2+}$</td>
<td>10</td>
<td>−73.60</td>
</tr>
</tbody>
</table>
93.3% to 109.2% were acceptable which indicated our method had good reliability of the sensing system.

Conclusions

In this paper, a fluorescence analysis method for the detection of VB$_{12}$ was established. The fluorescence suppression is attributed to inner filter effect because of the overlap between UV-vis absorption spectrum of VB$_{12}$ and emission/excitation spectra of CDs. There is a good linear relation between VB$_{12}$ concentration and ($F_0 - F$)/$F_0$ with the LOD is 93 nM. Compared with other method of fluorescence detection of VB$_{12}$, this method is green environment and less pollution. The detection system is simple and fast, the linear range is wider and the sensitivity is higher.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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References