Development of a sensitive B12 determination method based on inner filter effect on CdTe quantum dots

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Abstract

In this study, we developed a selective and sensitive inner filter effect-based method by thioglycolic acid capping CdTe quantum dots (TGA-CdTe QDs) for the quantitative determination of Vitamin B12 (B12). The effect of all experimental parameters and possible interference agents for determination of B12 by TGA-CdTe, were individually investigated and optimized. In addition, the mechanism of this detection method was discussed in detail. Under the optimum conditions, the fluorescence-quenching rate of the TGA-CdTe QDs showed a relative linear relationship against B12 concentration in the ranges of 0.02–0.4 and 1.5–70.0 μM B12 with a detection limit of 2.0×10^-8 M. Furthermore, the accuracy, precision and the practical application of determination of B12 by this developed method, were studied in pharmaceutical formulations injection without any sample pretreatment process. The percentage recoveries for B12 determination in these real samples were obtained in the range of 95.0%–105.0%.

Keywords: Vitamin B12, CdTe quantum dots, Inner filter effect, Fluorescence.

Introduction

Vitamin B12 with a carbon–metal bond (Figure 1), is a water-soluble vitamin that plays a critical role for formation, growing and repairing of all body cells and standard DNA synthesis. The deficiency of this vitamin can lead to pernicious anemia, cardiovascular disease, fatigue and weakness. Daily requirement dietary allowance of B12 for adults is adjusted 2.4 mg and 0.40 mg for children or pregnant and lactating women that can be naturally provided from foodstuff such as: meat, dairy products and egg. Therefore, B12 is recognized as an important molecule in medicine and nourishment and its monitoring is very important.

Until now, several methods have been reported to determine B12 such as capillary electrophoresis, high performance liquid chromatography (HPLC) with various detection methods such as UV/Vis, atomic absorption spectrometry, fluorescence, and mass spectrometric detectors, chemiluminescence methods, atomic absorption spectrometry, ultraviolet-visible (UV/Vis) spectrometry, voltammetry, radioisotope dilution, bioassay, mass spectrometric detection, enzyme-linked immunosorbed assays, microbiological assays, and radioassays. Unfortunately, some of these methods are often suffered from their limitations. However, development of an economical, easy, rapid, selective, and sensitive method for the quantitation specific determination of B12 is still very vital especially in complex samples such as pharmaceutical formulations and biological fluids. Fluorescence spectroscopy have shown high potential to improve sensitivity and selectivity of the existing analytical methods for determination of various analytes. However, since B12 is not naturally fluorescent, its can be determined by spectrophotometric methods through indirect methods. B12 can be also converted into fluorescent compounds, but this method is not usually reproducible and simple. Recently, semi-conductive quantum dots (QDs) by owning optical interested properties such as: high quantum yield and photostability, size-depending tunable photoluminescence, broad excitation spectra, narrow emission spectra and excellent chemical stability, have found wide application in fluorescence assays, biomedical, and material studies.

In addition, the wide excitation range of QDs have presented many wavelength choices to improve the sensitivity of the fluorescence-based methods with minimum background noises. Based on the above descriptions, in the present work, we succeeded to develop a sensitive, fluorescent method for B12 determination based on the inner filter effect (IFE) by the water-soluble CdTe QDs capped with thioglycolic acid (TGA). This approach could successfully offer considerable flexibility and more simplicity operation without needing any modification of QDs for linking by analyte. By introducing of B12 to the solution that contains TGA-CdTe QDs as probe, the emission intensity of the probe quenched in the shortest possible time, which can be calibrated to the added concentrations of B12.
Experimental

Materials and apparatus

Cd(NO\textsubscript{3})\textsubscript{2}, CdCl\textsubscript{2}, 2.5 H\textsubscript{2}O, NaBH\textsubscript{4}, Tellurium powder, potassium dihydrogen orthophosphate (KH\textsubscript{2}PO\textsubscript{4}) and dipotassium hydrogen orthophosphate (K\textsubscript{2}HPO\textsubscript{4}), citric acid, sodium citrate, tris for preparation of buffer, and thioglycolic acid (TGA) were purchased from Sigma chemical Company. NaCl, NaOH, Ethanol and other chemicals were purchased from Merck (Darmstadt, Germany, http://www.merck.de). The vitamins standards (B1, B2, B3, B6 and B12) were supplied from Amin pharmaceutical Company (Isfahan, Iran), which were used for preparation of the stock solutions of vitamins. All of the solutions (10\textsuperscript{3} mg L\textsuperscript{-1}) was prepared by dissolving 100 mg of each powder in 100 mL ultrapure water (resistance=18 M\Omega cm) and then stored in the dark at 4°C. Other used chemicals were of analytical grade without further purification. Phosphate buffers (20 mM) and other solutions were prepared with ultrapure Milli-Q water (resistance=18 M\Omega cm).

Equipment

All fluorescence measurements were performed by a Cary Eclipse spectrophotometer equipped with a quartz microcell (Agilent, USA, https://www.agilent.com). Both excitation and emission slits were set at 5 nm. UV-Vis spectra were recorded by an Agilent 8453 diode array spectrophotometer (Agilent, USA, https://www.agilent.com) over the range of 220-800 nm. All these optical measurements were repeated for three times (n=3) under ambient conditions. The morphology and the size of synthesized nanoparticles were studied by a Zeiss EM10C transmission electron microscope (Zeiss, Germany, https://www.zeiss.com). The TEM images of dried dropping casting solution on carbon coated copper grids, were captured at an accelerating voltage of 160 kV. In addition, a Malvern Dynamic Light Scattering (DLS) apparatus (Malvern, UK, https://www.Malvern.com) was used to investigate the size distribution of the synthesized nanoparticles.

Preparation of TGA capped CdTe QDs

The water-soluble CdTe QDs were synthesized according to a common reported procedure. Briefly, 0.1 gr Te powder was reduced by 0.280 gr NaBH\textsubscript{4} in 7 mL deionized water under stirring and nitrogen atmosphere. When the violet color of the solution was removed, the solution was ultra-filtered to remove the superfluous precipitate of NaBH\textsubscript{4}. The fresh prepared oxygen-free NaHTe aqueous solution was added into 200 mL nitrogen-saturated solution contained 0.358 gr CdCl\textsubscript{2}.2.5H\textsubscript{2}O and 0.2 mL TGA as a stabilizing agent. The pH of the mixture was adjusted at 10.0, and then transferred into a Teflon-lined stainless steel autoclave for heating at 90 °C in an oven for 3 hours. The obtained solution was diluted with ethanol to remove excess Cd\textsuperscript{2+} and TGA, and subsequently centrifuged at 4000 rpm for 15 min for three times. The obtained precipitate was dried under nitrogen atmosphere in a desiccator and then, dispersed in 250 mL ultrapure water as QDs mother solution. The concentration of the obtained TGA-CdTe QDs solution was evaluated as 6.67 µM according to a reported method. This solution was quite stable in the phosphate buffer (pH 7.4) at 4 °C in dark with no considerable changes in its optical characterization for 3 months.

Determination of B12 with functionalized TGA-CdTe QDs

1.0 mL of the synthesized TGA-CdTe QDs solutions was diluted 10 times with fresh phosphate buffer (pH=7.4) and mixed well. Various concentrations of stock solution of B12 was titrated manually on the diluted solutions of the CdTe-TGA QDs (2.0 mL) in a 1 cm×1 cm fluorescence quartz cell. After each addition of B12 and mixing for 5 min, the fluorescence intensity of the obtained solution was measured in the wavelength interval of 450–675 nm when 390 nm was radiated as excitation wavelength at room temperature.

Results and discussion

Characterization of synthesized TGA-CdTe QDs

To study the synthesized TGA-CdTe QDs, various methods (TEM, UV-Vis absorption, DLS and Fluorescence spectroscopy) were employed. The optical properties of QDs were studied using fluorescence and UV-Vis absorption spectrometry, Figures 2b. The TGA-CdTe QDs exhibited an excitation-independent photoluminescence (PL) behavior with relative narrow and symmetric emission spectra with a maximum PL intensity appeared at 523 nm, when was excited at 390 nm (with a full width at half-maximum about 58 nm). All these results confirmed the monodispersity and homogeneity of the synthesized QDs.

The fluorescence quantum yield (Φ) of the synthesized TGA-CdTe QDs was obtained to be 0.28 relative to fluorescein as reference dye with Φ of 0.93 at 490 nm and in sodium borate buffer (pH 9.5). The UV-Vis absorption spectrum of QDs exhibited an edge peak at about 500 nm, which indicated the average size of the TGA-CdTe QDs to be about 3.5 nm according to the reported method.

In addition, Figures 2a shows the TEM image of TGA-CdTe QDs which specified the roughly spherical nanoparticles with high dispersity and an average diameter of 3.6±4.0 nm. The DLS analysis shows an average size of 10 nm for TGA-CdTe QDs with a relative narrow dispersion (inset of Figures 2a) that supported the obtained TEM and UV-Vis spectroscopy results. It is notably that the DLS analysis estimates the hydrodynamic radius of TGA-CdTe QDs, but TEM image exhibits the electron rich area of each particle without any solvation layer which is equal by the dried particle size in colloidal suspension. Therefore, the obtain size from DLS is larger than TEM results.

Figure 1. The chemical structure of vitamin B12.
The effect of pH on the determination of B12 by TGA-CdTe QDs

Since the fluorescence behavior of QDs is directly dependent on the pH and the type of buffer, these factors were optimized to obtain high performance of the fluorescent probe for determination of B12. For this purpose, the effects of three type buffers including phosphate buffer, Tris–HCl buffer, and citrate buffer at the same pH were studied on the PL behavior of TGA-CdTe QDs. As shown in Figure 3a, the maximum relative fluorescence intensities of TGA-CdTe QDs in the absence of B12, was obtained when phosphate buffer (0.01M) was used. In addition, some solution of phosphate buffer with various pH were prepared to study the pH effect on the operation of B12 determination procedure by TGA-CdTe QDs.

As can be seen in Figure 3b, pH = 7.2 was obtained as the optimum pH for determination of B12 by TGA-CdTe QDs in phosphate buffer. Based on these results, the phosphate buffer solution (0.01M) with pH = 7.4 as selected as the optimum media phase for determination of B12 by QDs.

Method validation for quantification of B12 by TGA-CdTe QDs

For validation of the developed method, the fluorescence intensity of TGA-CdTe QDs was recorded in the presence of different concentrations of B12 under the optimum conditions. The obtained results are presented in the Figure 4a. As shown, a gradual decrease in the emission intensity of TGA-CdTe QDs happens by regular increasing the B12 concentration, which is due to absorption of TGA-CdTe emission by B12 as analyte. The concentration of B12 and the emission intensity can be successfully modeled by Stern-Volmer equation (Eq. 1).

\[
\frac{F}{F_0} = 1 + K_{qe} [Q]
\]  

Where F is the intensity of the QDs after addition of quencher concentration [Q] and F0 is the intensity of the QDs fluorescence in the absence of B12. The obtained experimental results for B12 determination by TGA-CdTe could be fitted linearly by conventional equation (Ln (F/F0) = 0.787C + 0.0376) in the range of 0.02–0.40 μM B12 with a correlation coefficient of R² = 0.9931, and “Ln (F/F0) = 0.395C + 0.4477” with a correlation coefficient of R²=0.9961 in the range of 1.50–70.0 μM of B12 (Figure 4b).

The limit of detections (LOD) of the method for determination of B12 was calculated to be 0.02 μM based on LOD (3.3σ/k), where σ is the standard deviation of the y-intercepts of the regression lines and (k) is the slope of the calibration graph. For more evaluation of this method, the merit figures of several last reported analytical methods were compared by theirs of this developed method (Table 1). It should be noted that despite the more superior detection limit of some of these reported methods compare to our developed IFE-based method, they often suffer from some limitations such as difficult operation and expensive equipment.

![Figure 2](image-url)  
**Figure 2.** a) TEM image and DLS histogram (inset) of the synthesized TGA-CdTe QDs, b) UV-visible absorption spectrum of B12, and UV-visible absorption and photoluminescence emission spectra of TGA-CdTe QDs.

![Figure 3](image-url)  
**Figure 3.** a) Depending of relative fluorescence of TGA-CdTe QDs to the kind of buffer media. b) Fluorescence intensity of TGA-CdTe QDs in the presence of B12 (40.0 μM) in phosphate buffer solutions with various pH values.
Study of the effect of foreign substances on the determination of B12 by TGA-CdTe

The possibility of the effects of few existence interferences that are existed in real samples, were investigated by measurement of the relative fluorescence intensity of TGA-CdTe QDs in the presence of 40.0 µM B12(F1) and coexisting substances at the same concentration (F2), Figure 5. The obtained result clearly confirmed the high practical ability of this developed method for determination of B12 in biofluids and other pharmaceutical formulations.

Determination of B12 in pharmaceutical formulations

Two pharmaceutical injection samples containing 1000 and 100 µg mL\(^{-1}\) of B12 were chosen as real samples to evaluate the practical ability of the proposed methods for B12 determination through standard addition method. The recovery rates and other figures of merit of this proposed methods for determination of B12 in these samples (1, 2) are shown in Table 2. The results indicated that the determination of B12 by TGA-CdTe could be satisfactorily employed for various forms of drug samples with any interferences.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Added (µM)</th>
<th>Expected (µM)</th>
<th>Found (µM)</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
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<tbody>
<tr>
<td>1</td>
<td>0.00</td>
<td>0.10</td>
<td>0.105</td>
<td>105</td>
<td>2.4</td>
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<td></td>
<td>5.00</td>
<td>5.10</td>
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<td>98</td>
<td>3.2</td>
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<tr>
<td>2</td>
<td>0.00</td>
<td>0.50</td>
<td>0.52</td>
<td>104</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>5.00</td>
<td>5.50</td>
<td>5.22</td>
<td>95</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Table 1. LOD of some reported analytical methods for the determination of B12 in real samples

<table>
<thead>
<tr>
<th>Method</th>
<th>LOD</th>
<th>Applications</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemiluminescence</td>
<td>5 pg mL(^{-1})</td>
<td>Pharmaceutical products</td>
<td>3</td>
</tr>
<tr>
<td>Enzyme-linked immunosorbert assay (ELISA)</td>
<td>0.0009 µg g(^{-1})</td>
<td>Food products</td>
<td>23</td>
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<tr>
<td>Radioisotopic assay</td>
<td>0.005 µg mL(^{-1})</td>
<td>Blood sample</td>
<td>26</td>
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<tr>
<td>Microbiological assay</td>
<td>0.03 µg g(^{-1})</td>
<td>Edible cyanobacteria</td>
<td>25</td>
</tr>
<tr>
<td>HPLC</td>
<td>0.04 µg mL(^{-1})</td>
<td>Multivitamin formulations</td>
<td>47</td>
</tr>
<tr>
<td>HPLC with atomic absorption spectrometry</td>
<td>42 µg mL(^{-1})</td>
<td>Meat and liver</td>
<td>48</td>
</tr>
<tr>
<td>HPLC with electrospray-ionization-mass spectrometry</td>
<td>0.002 µg g(^{-1})</td>
<td>Milk powder</td>
<td>49</td>
</tr>
<tr>
<td>Capillary electrophoresis-inductively-coupled plasma-mass spectrometry</td>
<td>0.05 µg mL(^{-1})</td>
<td>Multivitamin preparations</td>
<td>7</td>
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<tr>
<td>Biosensor assay based on- surface plasmon resonance</td>
<td>0.02 µg g(^{-1})</td>
<td>Infant formula</td>
<td>22</td>
</tr>
<tr>
<td>High performance capillary electrophoresis</td>
<td>20 µg mL(^{-1})</td>
<td>Corrinoid separation and determination</td>
<td>50</td>
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<tr>
<td>Multimode HPLC</td>
<td>0.025 µg</td>
<td>Vitamin B complex</td>
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<td>Reversed phase liquid chromatography-fluorimetry by a-ribazole as a marker</td>
<td>0.003 µg g(^{-1})</td>
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<td>Highly fluorescent vitamin B12 derivative</td>
<td>0.2–10 µg L(^{-1})</td>
<td>Foods (Beef, pork and cow’s milk)</td>
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<tr>
<td>Spectrophotometry</td>
<td>35 µg µL(^{-1})</td>
<td>Synthetic mixtures and vitamin B12 injections</td>
<td>51</td>
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<tr>
<td>TGA-CdTe fluorescent</td>
<td>0.027 µg mL(^{-1})</td>
<td>Vitamin B12 injections and artificial sample</td>
<td>This work</td>
</tr>
</tbody>
</table>

Figure 4. a) Fluorescence spectra of the CdTe-TGA in the presence of different concentrations of B12 from 0.02 to 70 µM. b) The Stern-Volmer plots of fluorescence intensity of CdTe-TGA at 523 nm against B12 concentration. 

Figure 5. Fluorescence intensity (F\(_1\)/F\(_2\)) of the TGA-CdTe QDs solution in the presence of B12 (40.0 µM) and several coexisting substances (40.0 µM). F\(_1\): The fluorescence intensity of the TGA-CdTe QDs solution in the presence of only B12. F\(_2\): The fluorescence intensity of the TGA-CdTe QDs solution in the presence of B12 and several coexisting substances.

Table 2. Determination of B12 by CdTe-TGA in pharmaceutical injection
Conclusion

In conclusion, we have employed the synthesized TGA-CdTe QDs for the assay of B12 over other coexisting substances in aqueous media. This determination method introduced here was developed based on IFE of B12 on the TGA-CdTe QDs due to a considerable spectral overlapping between absorption spectrum of B12 and emission spectrum of the QDs. Under optimized conditions, this method proved high sensitivity and selectivity for the quantitative determination of B12 assay in a broad linear range with a low detection limit. Furthermore, our nano-bio-probe successfully showed satisfactory recoveries for control of B12 level in pharmaceutical injection samples without needing complicated instruments and any separation step. This kind of IFE strategy is very simple compared to the complicated and costly process in quantitative determination of B12 and can be recommended as a simple, specific and urgent alternative method to solve the actual problem of the early diagnosis and prognosis monitoring of B12 without any boring separation and washing steps.

References