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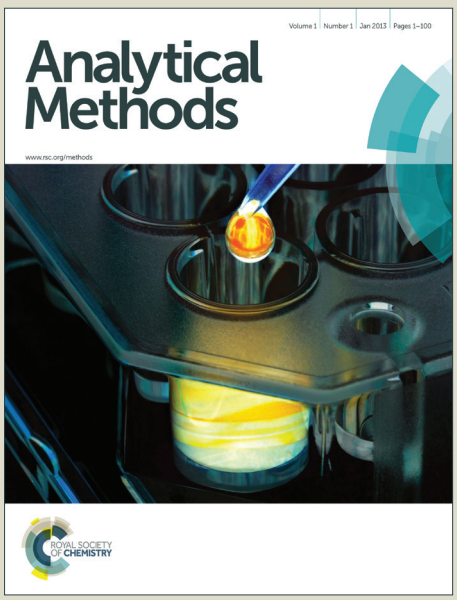

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Graphical abstract

for

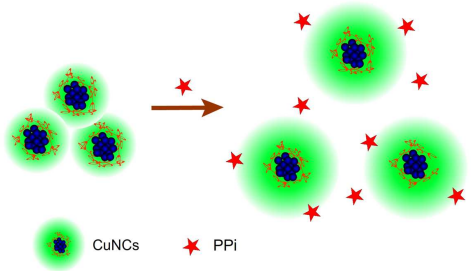
A new spectrofluorometric method for pyrophosphate assay based on the fluorescence enhancement of trypsin-stabilized copper clusters

Wei Wang,[a] Lei Zhan,[b] Yu Qing Du,[a] Fei Leng,[b] and Cheng Zhi Huang[a,b]*

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

[a] *Key Laboratory of Luminescent and Real-Time Analytical Chemistry (Southwest University), Ministry of Education, College of Pharmaceutical Sciences, Southwest University, Chongqing 400715, P. R. China. E-mail: chengzhi@swu.edu.cn, Tel: (+86) 23 68254659, Fax: (+86) 23 68367257.*
[b] *College of Chemistry and Chemical Engineering, Southwest University, Chongqing 400715, P. R. China.*

A new method for pyrophosphate detection in aqueous solution has been developed based on the fluorescence enhancement of trypsin-stabilized CuNCs.



Cite this: DOI: 10.1039/c0xx00000x

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PAPER

A new spectrofluorometric method for pyrophosphate assay based on the fluorescence enhancement of trypsin-stabilized copper clusters

Wei Wang,[a] Lei Zhan,[b] Yu Qing Du,[a] Fei Leng,[b] and Cheng Zhi Huang*[a,b]

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A novel and selective spectrofluorometric method for pyrophosphate (PPi) assay in aqueous solution was developed in this contribution on the basis of its enhanced fluorescence intensity of trypsin-stabilized copper clusters (CuNCs). Mechanism investigations showed that PPi can make the CuNCs dispersed, concomitantly resulting in the improved quantum yield and the enhanced fluorescence emission of the CuNCs. A good linear relationship in the range of 78 μM-20 mM was achieved between the enhanced fluorescence intensity of the CuNCs at 455 nm and the logarithm of PPi concentration with the detection limit (3σ) of 101 nM. By this approach, we can realized the purpose of simple, fast and inexpensive detection of PPi.

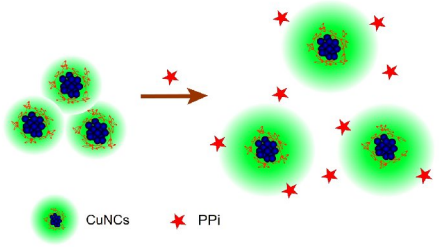
Introduction

Pyrophosphate (PPi) played an important role *in vivo* since it participated in a variety of metabolic reactions and bioenergetic processes such as the transhipment of intracellular calcium ion, the polymerization of DNA and RNA, the hydrolysis of adenosine triphosphate (ATP), the regulation of enzyme activity.[1-3] Abnormal secretion of PPi *in vivo* was closely related to some diseases such as arthritis and medial calcific sclerosis.[4] Therefore, to establish a novel method for the analysis of PPi in aqueous solution is of great importance.

There were various kinds of approaches for the analysis of PPi including fluorescence methods,[5-9] enzymatic methods[10] and colorimetric methods[11] and so on, in which the methods based on the coordination between metal ions and organic ligands were received extensive attention.[12-18] Specifically, most of the metal complex probes were developed based on the strong interactions between PPi and organic ligands coordinated metal ions. Such binding causes the alteration of the coordination complex. As a result, different optical signals were generated. However, the preparation of these probes was complicated, and some of the probes were not soluble in water. Furthermore, the steps of detection were relatively complex which greatly limited their applications in biological systems.

Metal nanoclusters, consisting of a few to tens of metal atoms with the size comparable to the Fermi wavelength of electrons, represent the missing link between metal atoms and nanoparticles and have molecule-like properties.[19] With the rapid development of nanotechnology, metal nanoclusters have received extensive attention due to their unique optical, physical, chemical, and electrical properties and they have been widely used in various fields.[20-25] Compared with gold nanoclusters (AuNCs) and silver nanoclusters (AgNCs), copper nanoclusters (CuNCs) were extensively regarded as promising nanomaterials for the reason that they also have the good performance of luminescence and enjoy many obvious advantages including highly catalytic efficiency and extremely low price. However, the preparation of ultra-small CuNCs was very difficult and they were susceptible to oxidation. In such case, there were still few reports about the synthesis and applications of CuNCs.[26-30] Recently, we developed a novel one-step synthesis of the trypsin-stabilized fluorescent CuNCs in aqueous solution without using additional protective or reducing agents, and the as-prepared CuNCs exhibited highly stable properties including oxidation resistance, thermal stability and photostability.[31]

Herein, for the first time, we established a novel turn-on fluorescent assay for PPi with these trypsin-stablized CuNCs (Scheme 1). It was found that the as-prepared CuNCs showed enhanced fluorescence emission at 455 nm when exited at 365 nm in the presence of PPi. Additionally, the enhanced fluorescence emission intensity has a good linear relationship to the logarithm of PPi concentration. Since these trypsin-stabilized CuNCs were easily prepared and water soluble, and showed a rapid response to PPi in aqueous solution, the method we proposed here is really simple, fast and inexpensive.



Scheme 1 Schematic illustration of a turn-on fluorescent assay of PPi.

Experimental

Reagents and chemicals

Trypsin was purchased from Aladdin Industrial Corporation (Shanghai, China). Sodium pyrophosphate (PPi) was purchased from Beibei Chemical Plant (Chongqing, China). $CuCl_2$ and other regents were of analytical grade without further purification before used. CH_3COOH-CH_3COONa buffers were used to control the acidity of the solution. Milli-Q purified water (18.2 MΩ) was used throughout.

Apparatus

The absorption spectra of trypsin and CuNCs were measured with an U-3010 spectrophotometer (Hitachi, Tokyo, Japan). An F-2500 spectrofluorometer (Hitachi, Tokyo, Japan) was used to measure the fluorescence intensity of the CuNCs before and after reaction with PPi. The morphology of the trypsin-stablized CuNCs was collected with a high resolution transmission electron microscopy (Tecnai G2 F20 S-TWIN, FEI, USA), which was operated with an accelerating voltage of 200 kV. An absolute photoluminescence quantum yield measurement system (Quantaurus-QY, Hamamatsu Photonics, Japan) with an excitation wavelength of 363 nm was used for measuring the absolute quantum yield (Φ) of the CuNCs. The circular dichroism (CD) spectra of the CuNCs were collected with a JASCO J-810 spectropolarimeter (Tokyo, Japan). A pH 510 precision pH meter was employed to measure pH values of buffers (California, USA). The fluorescence life time of the trypsin-stablized CuNCs was measured with an FL-TCSPC fluorescence spectrophotometer (Horiba Jobin Yvon Inc., France). A QL-901 vortex mixer (Haimen, China) was used for solution blending, and a high-speed H1650-W centrifuge (Xiangyi, China) was employed for the centrifugation of the solution.

Procedure

Preparation of the fluorescent CuNCs

The CuNCs were synthesized according to our previous report.[31] Briefly, $CuCl_2$ solution (2 mL, 100 mM) was added to trypsin solution (8 mL, 100 mg) by vigorous shaking for 3 min at room temperature. And then, the mixture was allowed to reflux for 12 h at 100 °C. The color of the solution changed from light blue to brownish yellow gradually indicating the formation of CuNCs. Finally, the CuNCs were purified by centrifugation (13000 rpm, 5 min) in order to remove the solid from the supernatant and stored at 4 °C before use.

General procedure for the detection of PPi

The determination experiments were performed by mixing 40 μL CuNCs and PPi solutions with various concentrations in CH_3COOH-CH_3COONa buffer (pH 5.2). Ultrapurified water was added to bring the final volume of 1000 μL and the mixture was incubate for 10 min at room temperature. The fluorescence intensity of CuNCs was measured on the F-2500 fluorescence spectrophotometer with an excitation wavelength of 363 nm.

Results and discussion

The characterization of the fluorescent CuNCs

To confirm the formation of CuNCs, the absorption and fluorescence emission were investigated. As shown in Fig. 1, the as-prepared CuNCs have an absorption band centered at 350 nm which was significantly different from the protein peak at 276 nm. Upon excitation at 363 nm, the trypsin-stablized CuNCs showed an emission band centered at 455 nm (Fig. 2) with an absolute fluorescence quantum yield of 1.1 % in water, the insert in Fig. 2 displayed the photographs of the 20-fold diluted CuNCs in water under the visible light (i) and 365 nm UV light irradiation (ii), respectively.

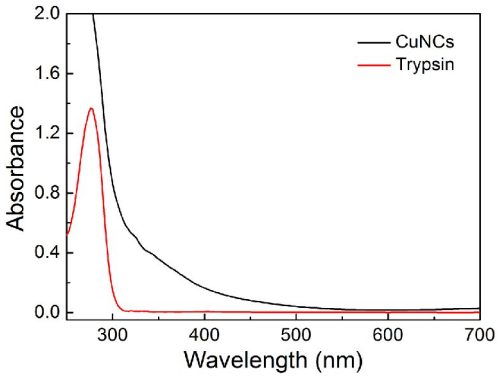


Fig. 1 The absorption spectrum of trypsin(red) and CuNCs(black).

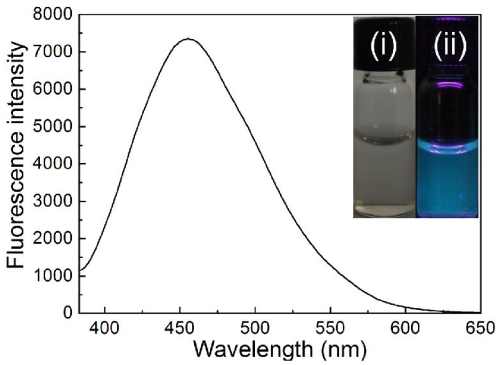


Fig. 2 The fluorescence emission spectrum of the trypsin-stabilized CuNCs with the excitation wavelength of 363 nm. Inset: the photographs of the 20-fold diluted trypsin-stablized CuNCs under the visible light (i) and 365 nm UV light irradiation (ii), respectively.

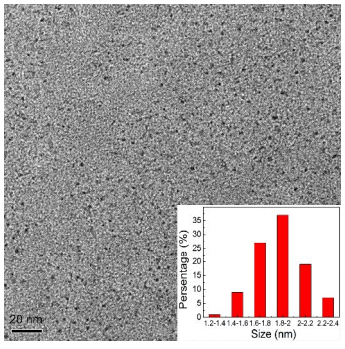


Fig. 3 TEM image of the CuNCs. Inset: the size distributions of CuNCs based on statistics of TEM image.

The water-soluble and blue-emitting trypsin-stablized CuNCs exhibited an average size of 1.87±0.53 nm according to the size statistics of 100 particles showed in the transmission electron microscopy (TEM) image (Figure 3).

Optimal conditions for the reaction between the CuNCs and PPi

First of all, the effect of acidity on the experiment has been studied (Fig. S1). It was reported that these trypsin-stabilized CuNCs were intrinsically pH sensitive and they exhibited strong fluorescence in acidic medium.[31] However, the fluorescence intensity of the CuNCs decreased gradually along with the increased pH values. Therefore, in this experiment, we chose CH_3COOH-CH_3COONa buffers to adjust the acidity of the solution. Specifically, the fluorescence intensity of the CuNCs was greatly decreased from pH 3.6 to 5.8. After adding a certain concentration of PPi, the fluorescence intensity of the CuNCs gradually increased and reached the maximum at pH 5.2. Subsequently, with the increasing pH values, the fluorescence intensity of the CuNCs was almost no longer increased. Therefore, we chose pH 5.2 in the following experiments.

In addition, we also investigated the effects of a series of reaction temperatures and times on this experiment (Fig. S2-S3). Surprisingly, the reaction temperature and time had little effect on this system, which demonstrated that the CuNCs probes can be applied for PPi analysis over a wide temperature (4-60 ^{o}C) and time (0-180 min) range. Therefore, room temperature was chosen in the subsequent experiments and the fluorescence intensity of the CuNCs was measured after 10 minutes reaction.

Fluorescent analysis of PPi

To explore the feasibility of this approach for quantitative analysis of PPi, fluorescence spectroscopy was used to investigate the response of the CuNCs toward PPi.

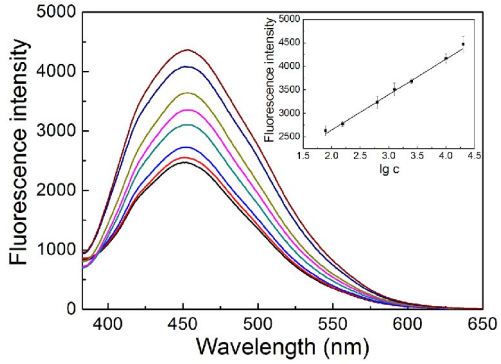


Fig. 4 Fluorescence emission spectrum of CuNCs in CH_3COOH-CH_3COONa buffer (pH 5.2) with the increasing concentrations of PPi: 0, 78.125 μM, 156.25 μM, 625 μM, 1250 μM, 2500 μM, 10000 μM, 20000 μM. Inset: The calibration curve of the logarithm of PPi concentration (lgc) versus the fluorescence intensity of the CuNCs at 455 nm. Excitation wavelength: 363 nm. Error bars represent standard deviations from three replicate measurements.

As shown in Fig. 4, after reacting with the increasing amounts of PPi in CH_3COOH-CH_3COONa buffer (pH 5.2), the fluorescence intensity of the trypsin-stabilized CuNCs was significantly enhanced. Notably, the enhanced fluorescence intensity was linearly proportional to the logarithm of the PPi concentration (lg c) from 78 μM to 20 mM with a corresponding correlation coefficient of 0.998. The linear equation was F = 752.7 lg c +1142.1 (F represents the fluorescence intensity of trypsin-stablized CuNCs and c represents the concentration of PPi), and the detection limit of 101 nM was achieved. The result indicated that this approach was applicable for quantitative analysis of PPi.

Selectivity of the CuNCs for PPi detection

To evaluate the specificity of the CuNCs toward PPi, we carried out the fluorescence experiment with other anions including F^-, Cl^-, Br^-, I^-, $H_2PO_4^-$, HPO_4^{2-}, HCO_3^-, NO_3^-, NO_2^-, CH_3COO^-, $B_4O_7^{2-}$, SO_4^{2-}, SO_3^{2-} under the identical conditions. As shown in Fig. 5, most anions have little effect on the fluorescence intensity of the CuNCs except HCO_3^-, NO_2^-, $B_4O_7^{2-}$ and SO_3^{2-} and these anions could quench the fluorescence of CuNCs to some extent. However, after adding PPi solutions with the same concentration to the aforementioned reaction system, it was clearly seen that the fluorescence intensity of the CuNCs was dramatic enhanced even in the presence of other anion. All these results indicated that there was no remarkable interference to the analysis of PPi in this system.

Additionally, the interaction between the trypsin-stabilized CuNCs and some polyphosphates were also investigated. However, these CuNCs also have a positive responce toward ATP, CTP, UTP GTP and hexametaphosphate. To be specific, the polyphosphates mentioned above can enhance the fluorescence intensity of CuNCs to a certain extent. Due to the different chemical structure of these polyphosphates and the complex conformation of the surface protein of the trypsin-stabilized CuNCs, the effect intensity between these polyphosphates and the CuNCs was not the same and the effect intensity of these polyphosphates toward the CuNCs was weaker than PPi. Therefore, in this scenario, the interference by these species must be taken into consideration and more effort should be made in developing assays based on the current probes.

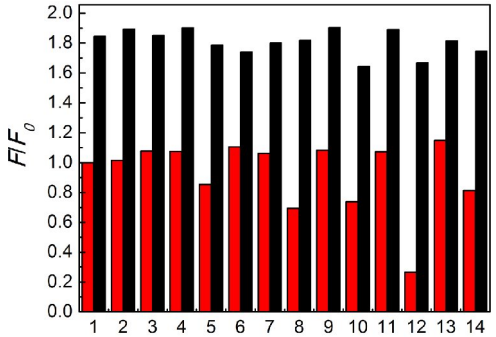


Fig. 5 The integrated fluorescence response upon addition of various anions (red bars, 10 mM) and the anions (10 mM) along with PPi (black bars, 10 mM) in CH_3COOH-CH_3COONa buffer (pH 5.2). 1, control; 2, F^-; 3, Cl^-; 4, Br^-; 5, I^-; 6, $H_2PO_4^-$; 7, HPO_4^{2-}; 8, HCO_3^-; 9, NO_3^-; 10, NO_2^-; 11, CH_3COO^-; 12, $B_4O_7^{2-}$; 13, SO_4^{2-}; 14, SO_3^{2-}. Excitation wavelength: 363 nm.

Investigations of the interaction mechanism between the CuNCs and PPi

To further investigate the mechanism for the fluorescence enhancement of the trypsin-stablized CuNCs, the conformational behaviors of the CuNCs before and after the reaction with PPi were observed by circular dichroism (CD) spectroscopy (Fig. 6). After reacting with PPi, the peak of the CuNCs at 197 nm was shifted to 193 nm, revealing that the conformation of protein has changed. Then, we evaluated the absolute fluorescence quantum yields (Φ) and fluorescence lifetime (τ_s) of the CuNCs after adding different concentrations of PPi and calculated the radiative decay rate constants (kr) from Φ and τ_s.[32,33] As shown in table S1, with the increasing concentration of PPi, the fluorescence lifetime of the CuNCs became shorter and the absolute quantum yields of the CuNCs were increased, which means the radiative decay rate constants were increased as well. This phenomenon indicated that the number of electrons of radiative transition were increasing, and therefore, the fluorescence intensity of the CuNCs was enhanced. In addition, we also characterized the morphology of the trypsin-stabilized CuNCs without and with PPi in CH_3COOH-CH_3COONa buffer (pH 5.2). From the images, we can clearly see that the trypsin-stabilized CuNCs became more dispersed after reacting with PPi (Fig. S3). Subsequently, the changes of average hydrodynamic diameters of the CuNCs were examined in CH_3COOH-CH_3COONa buffer (pH 5.2). It was found that the average hydrodynamic diameters of the CuNCs were decreased with the increasing concentration of PPi (Table S2) . To sum up, we believe that in the absence of PPi, since the CuNCs were dispersed in CH_3COOH-CH_3COONa buffer (pH 5.2), a little aggregation was appeared, thus the fluorescence intensity of CuNCs was relatively lower. After reacting with PPi, the dispersibility of trypsin-stabilized CuNCs was much better resulting in the enhanced fluorescence intensity of the CuNCs.

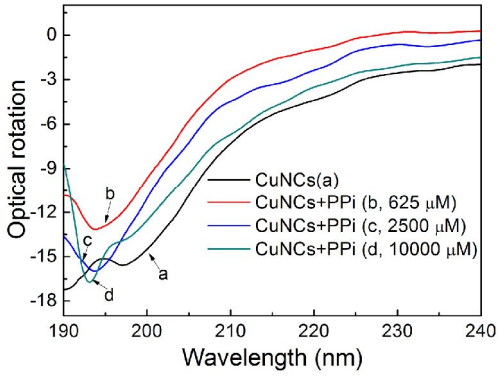


Fig. 6 Circular dichroism (CD) spectrum of the CuNCs before and after reacting with different concentrations of PPi in CH_3COOH-CH_3COONa buffer (pH 5.2).

Conclusions

In conclusion, we have successfully constructed a novel and simple method for the detection of PPi in aqueous solution based on the fluorescence enhancement of trypsin-stablized CuNCs. This method was extremely simple, fast and inexpensive. However, the sensitivity and selectivity need to be improved. We expect that the present findings will not only provide an significant clue to design fluorescent probes for PPi detection, but also open a door to the development of CuNCs for anions analysis.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (NSC, no. 21035005), the National Basic Research Program of China (973 Program, no. 2011CB933600) and the Cultivation Plan of Chongqing Science & Technology Commission for 100 Outstanding Science and Technology Leading Talents.

Notes and references

[a] *Key Laboratory of Luminescent and Real-Time Analytical Chemistry (Southwest University), Ministry of Education, College of Pharmaceutical Sciences, Southwest University, Chongqing 400715, P. R. China.*
[b] *College of Chemistry and Chemical Engineering, Southwest University, Chongqing 400715, P. R. China. Fax: +86-23-68254659; Tel: +86-23-68367257; E-mail: chengzhi@swu.edu.cn*

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