Autofluorescent Hyperbranched Poly(amide amine) as Effective Fluorescent Probe for Label-free Detection of Copper(II) Ions

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A label-free fluorescent probe based on autofluorescent hyperbranched poly(amide amine) (HPAMAM) for copper ions was designed. HPAMAM is a cationic polymer containing many amino groups, which could bind Cu²⁺ ions to form cupric amine complexes, leading to a selective quenching of the fluorescence intensity of HPAMAM via inner filter effect. The fluorescence intensity of HPAMAM decreased with increasing concentration of Cu²⁺ ions and the linear response ranged from 0.05 to 25 μM (R² = 0.995), with the corresponding detection limit (3σ/k) of 17.15 nM. The HPAMAM fluorescent probe provided a simple, rapid, selective and sensitive fluorometric method for detecting Cu²⁺ ions, which could be also applied for detection of Cu²⁺ ions in real water samples.

Keywords: HPAMAM, fluorescence intensity, cupric amine complex, copper(II) ions

(Received May 22, 2017; Accepted August 21, 2017; Published December 10, 2017)

Introduction

Copper plays an important role in environmental and biological fields, and is ranked as the third essential trace metal element in the human body and thus plays a fundamental role to maintain significant physiological processes. Copper deficiency may adversely affect enzyme activity and lead to a variety of neurological problems. On the other hand, an excess amount of copper can lead to gastrointestinal disturbance, liver or kidney damage. Moreover, misregulation of copper might cause human genetic disorders like Menkes’ and Wilson’s diseases; and neurodegenerative diseases such as Alzheimer’s and Huntington’s diseases. The U.S. Environmental Protection Agency (EPA) has set the limit for Cu²⁺ concentration in drinking water at ~20 μM. Due to the widespread use of copper in industrial and agricultural processes, Cu²⁺ contamination is quite a serious problem. Therefore, the development of a simple and rapid method for detecting Cu²⁺ is very important for human health and environmental protection. Different techniques, including surface plasmon resonance spectroscopy, atomic absorption spectroscopy, fluorescence spectroscopy, inductively coupled plasma mass spectroscopy, and electrochemistry for Cu²⁺ detection have been developed. Among these techniques, fluorescence spectroscopy has attracted considerable interest for its economy, high sensitivity, facile operation and rapid response.

Up to now, a number of fluorescent sensors based on quantum dots, metal nanoparticles, and fluorescent organic dyes have been employed to detect Cu²⁺. Nevertheless, these fluorescent probes suffer from some defects such as photobleaching of fluorescent dye, high toxicity and expensive cost of noble metal sources, which has further restricted their widespread application. Fortunately, it has been reported that the poly(amide amine) (PAMAM) dendrimers can emit strong fluorescence under suitable conditions and represent a new class of competitive material because of their autofluorescence and other prominent properties including favorable water solubility, biocompatibility, and non-immunogenic and environmental friendliness. They have been applied in different fields such as drug delivery, gene transfection and magnetic resonance imaging. However, dendrimers always demand stepwise synthesis and complicated purification. The tedious synthetic route is generally time-consuming and cost-enhancing. As is known to all, the structure and composition of hyperbranched polymers are similar to dendrimers, but they are usually prepared by a simpler and more economical one-pot polymerization technique. Moreover, the hyperbranched poly(amide amine) (HPAMAM) has been reported to emit striking fluorescence.

In particular, HPAMAM, a water-soluble, environmentally friendly and autofluorescent polymer without doping an external fluorochrome, is a cationic polymer containing many amino groups, and it has been used as polyvalence ligand to modify and stabilize some metal ions via chelation. It has been reported that HPAMAM can capture free copper(II) ions efficiently. In this work, we investigated the fluorescence responses of HPAMAM in the absence and presence of Cu²⁺ ions in detail. As shown in Scheme 1, Cu²⁺ ions could bind to the amino groups of the HPAMAM to form cupric amine complexes, resulting in the fluorescence intensity of HPAMAM quenching by the inner filter effect caused by the absorption of the excitation and/or emission light by absorbers in the detection system. Based on this, we designed a label-free, rapid, selective and sensitive fluorometric method to detect Cu²⁺ ions in aqueous media by using HPAMAM. Furthermore, the practicality of this novel method based on the HPAMAM probe to detect Cu²⁺ ions in real water samples also proved successful.

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Experimental

Reagents and chemicals
Diethylene triamine (DETA, 99%), methyl acrylate (MA, 98.5%), methanol (CH₃OH, 99%), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were obtained from Aladdin Chemistry Co., Ltd., China. Copper(II) sulfate pentahydrate (CuSO₄·5H₂O, >99%) was purchased from Beibei Chemical Reagents Factory (Chongqing, China) and all other chemicals not mentioned here were of analytical reagent grade and used without further purification. Milli-Q ultrapure water (18.2 MΩ·cm) was used throughout all the experiments.

Apparatus
The fluorescence excitation and emission spectra were recorded on an F-2700 fluorescence spectrophotometer (Hitachi, Japan). The slit (EX/EM) widths were 10 nm and 10 nm, and the (PMT) voltage was 400 V. The UV-vis absorption spectra in the wavelength range of 200 – 800 nm were obtained on a UV-2550 spectrophotometer (Shimadzu, Japan). 1H NMR spectra were recorded on a Bruker Avance III 600 MHz spectrometer (Bruke, Germany) using CDCl₃ as solvent. FTIR spectra were performed on a Nicolet 170SX spectrophotometer (PMT) voltage was 400 V. The UV-vis absorption spectra were measured on a Bruker Avance III 600 MHz spectrometer (Bruke, Germany) using CDCl₃ as solvent. FTIR spectra were measured with a Nicolet 170SX spectrophotometer (Madison, WI, USA). The pH values of solutions were measured with a pH meter (EL20, METTLER TOLEDO, Switzerland). The absolute quantum yield was recorded on a C11347 absolute PL quantum yield spectrometer (Hamamatsu, Japan).

Synthesis of hyperbranched poly(amide amine) (HPAMAM)

The HPAMAM was synthesized by a one-pot polymerization method described in a previous report. Briefly, the solution of diethylene triamine (10.42 g, 0.1 mol) and methanol (10 mL) was stirred in an ice-water bath. Next, methyl acrylate (10.49 g, 0.12 mol) was added dropwise into the reaction system under stirring. Methanol and excessive methyl acrylate were removed by a rotary evaporator under the vacuum after the reaction was kept for 48 h at room temperature. Then, the mixture was reacted for 1 h at 60°C, 2 h at 100°C, 2 h at 120°C and 2 h at 140°C using a rotary vacuum evaporator. The yellow product was dissolved in chloroform and precipitated in diethyl ether three times and dried at 40°C in a vacuum drying oven. 1H NMR spectra of HPAMAM were recorded (see Fig. S1, Supporting Information). HNMR (600 MHz, CDCl₃): δ = 1.88 – 2.45 (NH₃, NH₂), 2.45 – 2.94 (COCH₃, NHCH₃, NH₂CH₃), 3.12 – 3.51 (NCH₃), 3.58 – 3.71 (OCH₃), FTIR (KBr, Fig. S2, Supporting Information) ν/cm⁻¹: 3287 (NH₃), 3076 (NH), 2930 (CH₃), 2832 (CH₂), 1647 (C=O), 1555 (NH, CN), 1457 (CH₂), 1362 (NH, CN), 1276, 1122, 941, 750 (CH₂), 663 (NH₂). The 1H NMR and FTIR spectra were consistent with the previous report. The number-average molecular weight of HPAMAM was 10780 and polydispersity index (PDI) was 2.39, measured by Perkin Elmer HP 1100 gel permeation chromatograph (GPC).

Procedure for fluorometric detection of Cu²⁺

The fluorometric detection of Cu²⁺ was performed in HEPES (20 mM, pH 7.4) buffer solution at room temperature. Typically, the HPAMAM (final concentration 15 µg mL⁻¹), HEPES buffer (20 mM, pH 7.4) and different concentrations of Cu²⁺ were added into a colorimetric cylinder, then the mixtures were diluted to 5 mL with ultrapure water before being shaken thoroughly for 1 min. The fluorescence spectra of the mixtures were measured with the excitation wavelength at 270 nm. Here, fluorescence quenching efficiency is defined as the ratio ΔF/F₀ (ΔF = F₀ – F, ΔF is the change of fluorescence intensity of HPAMAM after the addition of Cu²⁺ ions), where F₀ and F are the fluorescence intensity of the HPAMAM in the absence and presence of Cu²⁺, respectively. All of the fluorometric detections were performed under the same conditions.

The Cu²⁺ assay of water samples

The water samples were collected from a local tap and boiled for 10 min, then, filtered with 0.22 µm water phase needle filter (Jin Teng) prior to the detection. Then the water samples were spiked with standard copper solution at different concentration levels (ultimate concentration: 0, 1, 10 and 20 µM). Finally, the spiked samples were measured according to the above detection procedures with the excitation wavelength at 270 nm.

Results and Discussion

Fluorescence response of the HPAMAM toward the Cu²⁺ and mechanism of fluorescence quenching

After adding Cu²⁺ ions into the HPAMAM solution, the striking blue fluorescence of HPAMAM could be remarkably quenched (see the inset of Fig. 1). The fluorescence spectra also indicated that the fluorescence intensity of the HPAMAM obviously decreased when Cu²⁺ ions were added. However, there was nearly no effect on the fluorescence wavelength (see Fig. 1). The UV-vis absorption spectra exhibited that the HPAMAM had an absorption band centered at 300 nm (curve a, Fig. 1). But there appeared two new absorption bands, one of which centered at 270 nm and the other was found in the range of 500 – 800 nm after adding Cu²⁺ ions into the HPAMAM (curve b, Fig. 1). It was worth noting that Cu²⁺ has only a very weak absorption band centered at 270 nm (curve c, Fig. S3, Supporting Information). Therefore, the two new UV-vis absorption bands could be owing to the cupric amine complexes that were formed from the amino groups of the HPAMAM in combination with the Cu²⁺ ions. Moreover, the absorption band centered at 270 nm had almost overlapped with the excitation spectra of the HPAMAM, and the broad absorption band ranging from 500 to 800 nm also had partial overlap with the emission spectra of the HPAMAM. It has been reported that the decreased fluorescence intensity of fluorophore was attributed to the absorption spectrum of the absorber, which has an overlap with the excitation and/or emission spectrum of the fluorophore.
Relationship between the fluorescence intensity and the concentration of HPAMAM. There was a good linear quenching of the fluorescence intensity of HPAMAM by the ethylenediaminetetraacetate (EDTA) was added into the emission (e and f) spectra of 150 μg mL⁻¹ HPAMAM solution in the absence (i) and presence (ii) of 250 μM copper ions under UV lamp (excitation at 365 nm).

Fig. 1 The UV-vis absorption (a and b), excitation (c and d), and emission (e and f) spectra of 150 μg mL⁻¹ HPAMAM in the absence (a, c and e) and presence (b, d and f) of 250 μM Cu²⁺ ions. Insert: the photographs of 150 μg mL⁻¹ HPAMAM solution in the absence (i) and presence (ii) of 250 μM copper ions under UV lamp (excitation at 365 nm).

Fluorescence quenching efficiency (ΔF/F₀) of 15 μg mL⁻¹ HPAMAM + 25 μM Cu²⁺ at different pH values (pH 7.0 – 8.0). (Fig. 2)

Fig. 2 Fluorescence quenching efficiency (ΔF/F₀) of 15 μg mL⁻¹ HPAMAM + 25 μM Cu²⁺ at different pH values (pH 7.0 – 8.0).

fluorophore, i.e., inner filter effect. The amino groups of the HPAMAM were able to interact with Cu²⁺ to form cupric amine complexes that acted as an absorber, leading to a selective quenching of the fluorescence intensity of HPAMAM by the inner filter effect.

In order to investigate the mode of interaction between Cu²⁺ ions and the HPAMAM, we designed the assay that the ethylenediaminetetraacetate (EDTA) was added into the HPAMAM-Cu²⁺ complex system. And the result exhibited that the quenched fluorescence of the HPAMAM by Cu²⁺ can be basically recovered through a stronger competitive complexation reaction (Fig. S4, Supporting Information), which indicated that the interaction between Cu²⁺ and the HPAMAM was through complexation reaction.

**Optimum conditions for the fluorescent probe**

The HPAMAM had two excitation peaks, with one centered at 242 nm and the other centered at 270 nm (curve c, Fig. 1). The effect of different excitation wavelengths on the fluorescence quenching efficiency (ΔF/F₀) were studied (Fig. S5, Supporting Information). The values of (ΔF/F₀) for the excitation wavelength at 242 and 270 nm were 63.6 and 70.1%, respectively. This result showed that a higher sensitivity could be obtained with the excitation wavelength at 270 nm, which was chosen in our detection system.

The concentration of HPAMAM should be optimized before investigating the fluorescence response of the HPAMAM for detection of Cu²⁺. From the relationship between the fluorescence emission intensity and the concentration of HPAMAM (Fig. S6, Supporting Information), it could be found that the fluorescence emission intensity depended on the concentration of HPAMAM. There was a good linear relationship between the fluorescence intensity and the concentration of HPAMAM in the range of 5 – 100 μg mL⁻¹. However, when the concentration of HPAMAM was higher than 100 μg mL⁻¹, the fluorescence intensity deviated from linearity and was relatively insensitive to the change of the concentration of HPAMAM. Therefore, the optimal concentration of HPAMAM should be chosen in the sensitive and linear response range that was 5 – 100 μg mL⁻¹. We carefully investigated the variation of the fluorescence quenching efficiency (ΔF/F₀) depending on the concentration of HPAMAM in the range of 5 – 40 μg mL⁻¹ in the presence of 25 μM Cu²⁺ ions. Generally, in the presence of a quencher with a given concentration, fluorescent probe with lower concentration was found to possess higher quenching efficiency (ΔF/F₀), which means higher sensitivity. As shown in Fig. S7 (Supporting Information), the fluorescent probe with lower concentration was found to possess higher ΔF/F₀ when the concentration of fluorescent probe in the range of 15 – 40 μg mL⁻¹ and the maximal (ΔF/F₀) was obtained when the concentration of HPAMAM was 15 μg mL⁻¹. However, the ΔF/F₀ gradually decreased with the decreasing of the concentration of HPAMAM in the range of 15 – 5 μg mL⁻¹, which might result from the decrease in the signal-noise ratio of instrumentation when the concentration of fluorescent probe was too low. Therefore, 15 μg mL⁻¹ of HPAMAM was eventually chosen in this study.

In order to achieve the highly sensitive detection of Cu²⁺ by using HPAMAM, the effect of pH correlated to the fluorescence intensity was studied and optimized. Britton-Robinson (BR) buffer was used in a pH screening test, owing to the feature that BR buffer covers the wide pH range between 1.8 and 11.9. The fluorescence intensity of HPAMAM and HPAMAM-Cu²⁺ complex at different pH were tested (Fig. S8A, Supporting Information). It could be known that the fluorescence intensity of HPAMAM gradually decreased with the increasing of pH in the range of 2 to 11. It was reported that the strong charge-charge repulsion and the strength of the hydrogen bond in low pH range made the HPAMAM structure more rigid and co-planar, resulting in improving the fluorescence efficiency. However, the fluorescence quenching efficiency (ΔF/F₀) gradually enhanced with increasing pH then reached a stable level (Fig. S8B, Supporting Information). When Cu²⁺ ions were added in acidic media (pH ≤ 4.0), the fluorescence quenching efficiency (ΔF/F₀) had no significant change. It might be due to the fact that the amino groups of the HPAMAM were well protonated so that they were unable to combine with Cu²⁺ to form cupric amine complexes. Due to un-ionized formation among the amino groups of HPAMAM in an alkaline environment (pH ≥ 7.0), there were almost no electrostatic repulsions, so the amino groups could bind Cu²⁺ to form cupric amine complexes. The fluorescence intensity of HPAMAM-Cu²⁺ complex with the pH in the range of 7.0 to 8.0 also was tested (Fig. 2). The results showed that the fluorescence
quenching efficiency ($\Delta F/F_0$) reached a maximum value and nearly remained constant at pH $\geq 7.4$. Given that the fluorescence intensity of HPAMAM gradually decreased with increasing of pH (Fig. S8A, Supporting Information) and that the higher the fluorescence intensity of HPAMAM, the wider the linear range of detection, pH 7.4 was chosen in this detection system. Meanwhile, the absolute quantum yield of HPAMAM was 2.1% when the pH value was 7.4.

To find a suitable medium for achieving good sensitivity and reproducibility of the response, the detection of Cu$^{2+}$ using the HPAMAM probe in four different buffer systems (20 mM, pH 7.4), namely PBS, BR, HEPES and Tris–HCl, were carefully tested. As shown in Fig. S9 (Supporting Information), the fluorescence quenching efficiency ($\Delta F/F_0$) for PBS, BR, HEPES and Tris–HCl were 67.4, 68.2, 70.1, and 58.1%, respectively. The results demonstrated that the fluorescence quenching efficiency ($\Delta F/F_0$) obtained in the HEPES buffer was the best among the buffers. This is probably because of the presence of tris(hydroxymethyl)aminomethane (tris) in Tris–HCl buffer and the presence of phosphate in PBS and BR buffer. When Cu$^{2+}$ ions were added to Tris–HCl, PBS and BR buffer, respectively, it could form Cu(II)-Tris complexes or cupric phosphate, which is poorly soluble or slightly soluble in water. That would prevent Cu$^{2+}$ ions from combining with the amino groups of the HPAMAM to form cupric amine complexes. Therefore, HEPES buffer was selected for this work.

Time response of the HPAMAM-Cu$^{2+}$ complex was studied as shown in Fig. S10 (Supporting Information). It could be known that the fluorescence quenching efficiency ($\Delta F/F_0$) reached the maximum within 1 min and remained stable in the following 1 h of observation after adding Cu$^{2+}$ ions. The result indicated that the reaction between HPAMAM and Cu$^{2+}$ was fast and stable, suggesting it would be a promising probe for rapid detection of Cu$^{2+}$ without strict time demand. Therefore, the fluorescence intensity in our detection system was recorded at 1 min after adding the Cu$^{2+}$ ions.

### Sensitivity and selectivity of the HPAMAM for detection of Cu$^{2+}$

To evaluate the sensitivity of the HPAMAM for detecting Cu$^{2+}$, under optimum conditions, the fluorescence emission spectra at 392 nm was monitored after adding different concentrations of Cu$^{2+}$ (Fig. 3). The change of fluorescence intensity $\Delta F$ was highly dependent on the quantity of Cu$^{2+}$ that was added. It was clearly seen that a good linear relationship between the $\Delta F$ and the concentration of Cu$^{2+}$ ranged from 0.05 to 25 $\mu$M ($R^2 = 0.995$). But the fluorescence response deviated from linearity when the concentration of Cu$^{2+}$ was
higher than 25 μM. The corresponding linear equation was 
\[ \Delta F = 6.388C + 3.789, \]  
where C (μM) represents the concentration of Cu\textsuperscript{2+}. According to IUPAC recommendations, the limit of detection (LOD) was calculated to be 17.15 nM based on 3σ/k, where σ is the standard deviation of the blank signals of HPAMAM, which was much lower than the maximum allowable level of Cu\textsuperscript{2+} (ca. 20 μM) in drinking water set by the US EPA. These results showed that HPAMAM is a very promising probe for detection of Cu\textsuperscript{2+}.

Selectivity is another important indicator of evaluating the properties of the probe. The values of (ΔF/ΔF₀) of the HPAMAM system in the presence of the representative metal ions including Ca\textsuperscript{2+}, Cd\textsuperscript{2+}, Co\textsuperscript{2+}, Fe\textsuperscript{3+}, Mg\textsuperscript{2+}, Mn\textsuperscript{2+}, Zn\textsuperscript{2+}, Pb\textsuperscript{2+}, Ag\textsuperscript{+}, Na\textsuperscript{+}, K\textsuperscript{+}, Fe\textsuperscript{3+}, Ba\textsuperscript{2+} and Cu\textsuperscript{2+} under the same conditions were measured (Fig. 4A). It was found that only Cu\textsuperscript{2+} caused prominent quenching of the fluorescence emission intensity. What is more, an interference study was performed to further investigate the specificity of the HPAMAM system in the presence of other coexisting ions (Fig. 4B). It revealed that the high selectivity of the fluorescence probe toward Cu\textsuperscript{2+} ions in the presence of other coexisting metal ions was still present. The above results further proved that HPAMAM was highly specific toward Cu\textsuperscript{2+}. This excellent specificity arose from the fact that amino groups of HPAMAM could bind Cu\textsuperscript{2+} ions to form cupric amine complexes, leading to a selective quenching of the fluorescence intensity of HPAMAM via inner filter effect. These results indicated that the HPAMAM probe could be applied for Cu\textsuperscript{2+} ion detection in real water samples.

Application in real sample analysis

On the basis of the above results, the probe was applied to detection of Cu\textsuperscript{2+} in tap water samples. We used a standard addition method to measure the concentration of Cu\textsuperscript{2+} in water samples. As shown in Table 1, the recoveries of tap water were 97.1 – 103.9% and the relative standard deviation (RSD) values were in the range of 1.07 – 2.34%, which was obtained by repeating the experiment five times under the same conditions. The results of the recovery value and RSD were satisfactory, indicating that the method using the HPAMAM probe to detect Cu\textsuperscript{2+} ions in real water samples was reliable.

Conclusions

In this work, the autofluorescence HPAMAM was prepared by a simpler and more economical one-pot polymerization technique. The amino groups of the HPAMAM could interact with Cu\textsuperscript{2+} to form an absorbent, resulting in a selective quenching of the fluorescence intensity of HPAMAM by inner filter effect. HPAMAM was found to be an excellent probe for Cu\textsuperscript{2+} detection. HPAMAM as fluorescent probe showed many advantages, including rapid detection, simple operation, good selectivity, lab-free and low cost. Overall, these results demonstrated that the HPAMAM system has great potential in environmental and biological sample analysis.

Supporting Information

\'H NMR and FTIR spectra of HPAMAM; UV-vis absorption spectra of the mixture of (a) 150 μg mL\textsuperscript{-1} HPAMAM and 250 μM Cu\textsuperscript{2+} ions, (b) 150 μg mL\textsuperscript{-1} HPAMAM and (c) 250 μM Cu\textsuperscript{2+} ions; fluorescence emission spectra of 15 μg mL\textsuperscript{-1} HPAMAM in the absence (a) and presence (b) of 25 μM Cu\textsuperscript{2+} ions, and (c) in the presence of 25 μM Cu\textsuperscript{2+} ions and 25 μM EDTA; optimization of experimental conditions are listed in the Supporting Information. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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