Fluorescent Sensor for Melamine Based on Its Copper Complex Interrupted of AT-dsDNA Copper Nanoparticles

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Abstract A novel turn-off strategy was developed for the melamine (Mel) detection based on melamine-Cu conjugate and subsequent interrupted the formation of AT-dsDNA copper nanoparticles (AT-dsDNA CuNPs). The coordination of melamine to copper cannot make the reduction of Cu^{2+} to Cu^{0} enough in the AT24-CuNPs synthesis process, and thereby results in the fluorescence intensity of CuNPs decreasing. Under the optimum conditions, the concentration of melamine showed a good linear relation with the fluorescence response of CuNPs in the range of 1～150 $\mu mol \cdot L^{-1}$. The detection limit was 0.5 $\mu mol \cdot L^{-1}$. Furthermore, good recoveries were obtained while the proposed method was applied to the analysis of melamine in milk samples.

Keywords Copper nanoparticles; Cu-Mel complex; Label-free sensor; Melamine detection

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Introduction

It is -clear that melamine (Mel) is a nitrogen-rich compound which has been illegally added into milk products and animal feeds for a higher indication of protein content. Although melamine has low toxicity, melamine in the human body can form the insoluble crystals which cause serious harm to human health, including urinary calculi, kidney stones, and even death[1]. Therefore, the development of sensitive, robust and selective strategies for detecting melamine is of great significance for food safety, environmental monitoring and fundamental research.

Conventional methods for the detection of melamine include chromatography-mass spectrometry[2], high performance liquid chromatography, gas chromatography. These assays, however, are laborious, time-consuming and expensive, which restrians them from a wide applicaiton. More recently, many new strategies have been developed for melamine analysis. Examples are electrochemical approaches, colorimetric detection[3-5], fluorescence detection[6], Mg^{2+}-dependent DNAzymes, molecularly imprinted polymer[7], surface enhanced Raman scattering spectroscopy[8-9]. Despite the powerful of these techniques, the need for high limits of detection, complicated pretreatment samples, high enzyme cost or prepared of polymers is still limited. Thus, a simple strategy with rapid operation at low cost for detection of melamine is still in great desired.

Very recently, fluorescent copper nanoparticles (CuNPs) have induced significant interest due to their easy synthesis, strong fluorescence and good biocompatibility. Extensive sensors based on CuNPs as fluorescent probes have been successfully employed for proteins[10], small molecules[11] and enzyme assays[12]. In particular, Wang's and Ouyang's group report that poly(AT-TA) can be employed as the specific sequence template for CuNPs synthesis[13-14]. The poly(AT)-CuNPs show remarkable fluorescence response compared with random sequences dsDNA-CuNPs or poly(T)-CuNPs. In addition, the fluorescence intensity and fluorescence lifetime of CuNPs can be regulated by the length of poly AT. Moreover,

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the poly(AT)-CuNPs are synthesized within several minutes, which is much faster and more convenient than other fluorescent metal nanoparticles, such as random sequences dsDNA-CuNPs (>1 h for efficient hybridization), silver nanoclusters (24 h in the dark at 4 ℃), and gold nanoclusters (2 days). Thus, poly(AT)-CuNPs suit well as a fluorescent probe for chemical analysis.

Melamine has been reported to coordinate with Cu^{2+} via aromatic nitrogen atom to form Cu-Mel complex[15]. We are considering if it is possible that melamine can hinder the synthesis of CuNPs though the interaction between melamine and Cu^{2+}. That is to say, for the interaction of melamine to Cu^{2+} there is not enough reduction of Cu^{2+} to Cu^0. Hence, the formation of CuNPs is interrupted by melamine. Enlightened by this idea, a fluorescence detection of melamine is designed during the formation of CuNPs. It was found that the presence of the melamine has weaken the fluorescence of CuNPs. The detection of melamine can be achieved during the formation of CuNPs. As best of our knowledge, this is the first example of detection of melamine by using the coordination of melamine to Cu^{2+} as hamper for the generation of CuNPs. With this proposed method, melamine in milk products can be detected simplely, easily and efficiently.

1 Experimental

1.1 Reagents and materials

Purified oligonucleotides were synthesized from Takara Biotechnology Co. Ltd. (Dalian, China). 3-(N-morpholino)-propane sulfonic acid (MOPS), $CuSO_4 \cdot 5H_2O$, (+)-sodium ascorbate, melamine were purchased from Sangon Biotechnology Co. Ltd. (Shanghai, China). The MOPS buffer (10 mmol·L^{-1} MOPS, 150 mmol·L^{-1} NaCl, pH 7.6) was used for the formation of fluorescent CuNPs. All reagents were analytical grade and solutions were prepared with ultrapure water (electric resistance >18.3 MΩ). Liquid milk was purchased from local supermarkets.

Fluorescence measurements were recorded on Cary Eclipse fluorescence spectrometer (Agilent, USA) in a wavelength ranging from 400 to 660 nm. The excitation wavelength was set at 340 nm. The excitation and emission slits was set at 5 nm and 10 nm, respectively. All measurements were conducted at room temperature [(20±1) ℃].

1.2 Synthesis of AT24-CuNPs

AT24 CuNPs were synthesized according to previous reports with a slight modification[13]. Firstly, 200 nmol·L^{-1} ds-DNA was mixed in MOPS buffer (10 mmol·L^{-1} MOPS, 150 mmol·L^{-1} NaCl, pH 7.6). The mixture was incubated at room temperature for 15 min to ensure complete hybridization interaction. Then 2 mmol·L^{-1} sodium ascorbate and 300 μmol·L^{-1} $CuSO_4$ was added into the mixture solution and incubated for 2 min at room temperature in the dark to form AT24 CuNPs.

1.3 Fluorescence detection of melamine

For melamine detection, different concentration of melamine was pre-incubated with 300 μmol·L^{-1} $CuSO_4$ for 20 min. Then, the mixture was added into a reaction solution containing 200 nmol·L^{-1} ds-DNA and 2 mmol·L^{-1} sodium ascorbate. After the incubation for minutes, the resulting solution was immediately subjected to fluorescence measurement.

2 Results and Discussion

2.1 Design of the detection scheme

Previously, Wang's and Ouyang's research groups have reported that ds(AT-TA) can act as an efficient template for forming the fluorescent CuNPs and show remarkable fluorescence response compared with other nonspecific sequence[13-14]. On the other hand, it was reported that the melamine could coordinate with Cu^{2+} to form Cu-Mel complex through one aromatic nitrogen atom when [Cu^{2+}] > [Mel][15]. Our strategy is based on an inhibition of AT24-CuNPs by Mel. With the presence of Mel, Mel bonds with Cu^{2+} and the reduction of Cu^{2+} to Cu^0 in the AT24-CuNPs synthesis process does not occur, which results in a low fluorescence signal (Fig. 1). Therefore, the Cu-Mel complex shows a sensitive fluorescence response that can be used for reliable detection of Mel.

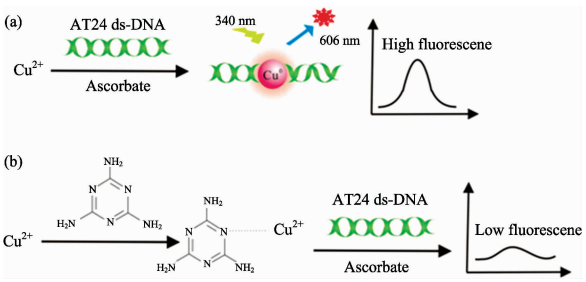

Fig. 1 (a) The formation procedure of AT24-CuNPs and (b) the process that melamine hinders the generation of AT24-CuNPs

2.2 Evaluation of Mel inhibition on Cu NPs formation

To check whether the melamine could inhibit the synthesis of CuNPs indirectly, the fluorescence spectra of the CuNPs in the presence or absence of melamine were firstly collected and shown in Fig. 2. Negligible fluorescence intensity was observed in the absence of Cu^{2+} (curves *a*). Conversely, a strong fluorescence signal at 606 nm was observed once in the presence of Cu^{2+} (curves *b*), suggesting the formation of CuNPs effectively at a low concentration of Cu^{2+} though

reduction by sodium ascorbate. As expected, once Cu^{2+} was pretreated with melamine, a remarkable fluorescence decreasing appeared (curves *c*). These results implied that CuNPs synthesis was inhibited though the formation of the stable complex between melamine and Cu^{2+}. A control experiment was performed by adding melamine after accomplishing the synthesis of CuNPs. Even a moderate change of fluorescence intensity confirmed the negligible effect of melamine on the as-prepared CuNPs (curves *d*). Therefore, the proposed strategy was proved to be feasibly for the detection of melamine.

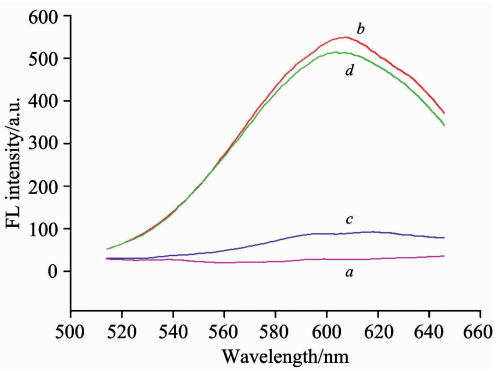

Fig. 2　Fluorescence emission spectra of CuNPs under different conditions

a: dsDNA+ascorbate; *b*: (dsDNA+ascorbate)+Cu^{2+}; *c*: (dsDNA+ascorbate)+(Cu^{2+}+Mel); *d*: (dsDNA+ascorbate)+Cu^{2+}+Mel; Cu^{2+}: 300 μmol·L^{-1}; ascorbate: 2 mmol·L^{-1}; Mel: 150 μmol·L^{-1}

2.3　Optimization of the reaction condition

In order to obtain a high effective analysis performance for the detection of melamine, several factors including reaction time between Cu^{2+} and melamine, the amount of Cu^{2+}, pH, time of CuNPs synthesis was investigated.

Fig. 3(a) shows the effect of reaction time between Cu^{2+} and melamine. It can be seen that the fluorescence intensity decrease significantly with 20 min, and then reach a platform after 20 min, indicating that 20 min was sufficient to complete the interaction between Cu^{2+} and melamine. Therefore, a period of 20 minutes was selected for the next experiments.

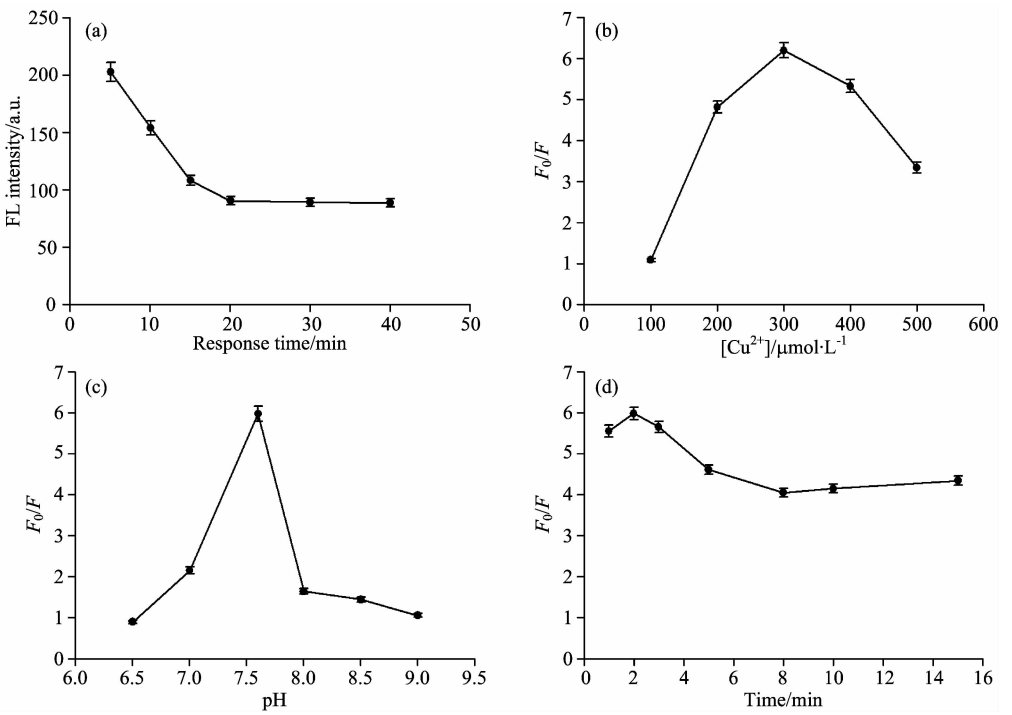

Fig. 3　Effects of (a) reaction time between Cu^{2+} and melamine; (b) amount of Cu^{2+}, (c) pH, (d) time of CuNPs synthesis on the fluorescence response of the sensor for melamine detection

Previous reports showed that Cu^{2+} plays an important role in the formation of CuNPs. The optimal of Cu^{2+} concentration may be related to characteristics of CuNPs synthesis. Low concentration of Cu^{2+} is disadvantageous for preparation of CuNPs. However, if the Cu^{2+} concentration is too high, DNA template would be degraded by oxygen-based radicals produced at high Cu^{2+} concentration and result in fluorescence intensity decreasing. Therefore, the effect of Cu^{2+} concentration in the range of 100～500 μmol·L^{-1} was studied [Fig. 3(b)]. An excellent signal-to-background ratio F_0/F (F_0 and F are the fluorescence intensity in the absence or presence of melamine) was obtained when the concentration of Cu^{2+} was 300 μmol·L^{-1}. As a result, 300 μmol·L^{-1} was selected as the optimum concentration.

It has been found that the formation of CuNPs was obvious pH depended and relatively low in acidic solution. Therefore, the effect of pH on the fluorescence response was optimized [Fig. 3(c)]. It is clear that F_0/F is supposed to be observed at the highest value at pH 7.6. So MOPS at pH 7.6 is used for the following experiments.

The time of CuNPs formation is a key point that affects fluorescence assays, and the relationship between reaction time and fluorescence ratio (F_0/F) is examined. As shown in Fig. 3(d), the maximum F_0/F is obtained at 2 min. Therefore, a further assay takes two minutes.

2.4 Analytical performance

Under the optimal assay conditions, quantitative analysis of melamine was evaluated. Fluorescence emission spectra of CuNPs in the presence of different concentration of melamine are shown in Fig. 4(a). It was observed that the fluorescence intensity of the CuNPs decreased gradually with increasing melamine concentration. A good liner correlation to melamine concentration was obtained range from 1 μmol·L^{-1} to 150 μmol·L^{-1} with a correlation coefficient of 0.999, as shown in Fig. 4(b). The detection limit was estimated to be 0.5 μmol·L^{-1} in terms of the 3σ rule, which was lower than that of the previously reported sensors (Table 1)[3-6, 8-9]. It is evident that the proposed method is more cost effective, simple with less detection limit of melamine compared to silver nanoparticles, quantum dots, gold nanoparticles reported earlier.

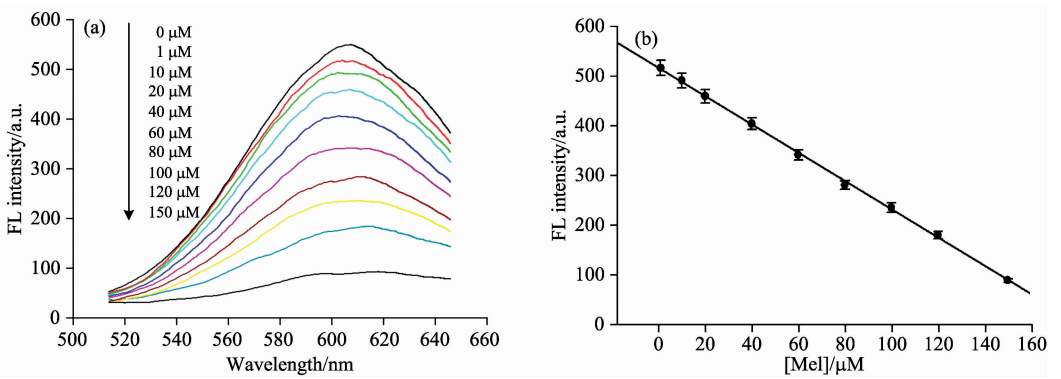

Fig. 4 (a) Fluorescence spectra of the sensor in response to melamine of various concentrations; (b) Plot of fluorescence intensity of the CuNPs versus melamine concentration. Error bars are standard deviation across three repetitive experiments

Table 1 Comparison of our proposed method with some previously reported melamine sensors

Tool	Method	Detection limit/(μmol·L^{-1})	Ref.
Au nanoparticles	Colorimetry	7.9	[3]
label free AgNPs	Colorimetry	2.32	[4]
cyclodextrin-capped AgNPs	Colorimetry	4.98	[5]
cucurbit[7]uril	fluorescence	1.5	[6]
silver nanoparticles	SERS	3.96	[8]
gold nanoparticles	Colorimetry and SERS	2	[9]
AT-dsDNA CuNPs	Fluorescence	0.5	This work

The selectivity of the proposed method was also studied by using other interferences possibly existing in dairy products such as glycine (Gly), glucose (Glc), aniline (Ani), lysine (Lys), histidine (His) and common ions. It was clearly revealed that only melamine induced a dramatic decrease of the fluorescence, whereas no apparent fluorescence changes were observed for most other interferences (Fig. 5). Although histidine also showed some response due to the imidazole nitrogen of histidine, the decrease of the fluorescence was much lower than that of melamine. Combining appropriate pretreatment and separation technology, the interferences of aromatic nitrogen atoms compounds would be avoided in the fluorescence analysis of melamine in milk samples.

2.5 Determination of melamine in real samples

The practical application of the proposed method was performed by determination melamine in spiked milk using a standard addition method. The results are listed in Table 2. No melamine was found in spiked milk samples. And good recoveries of melamine were obtained range from 95.0 to 100.4% indicating the reliability of proposed method in real applications.

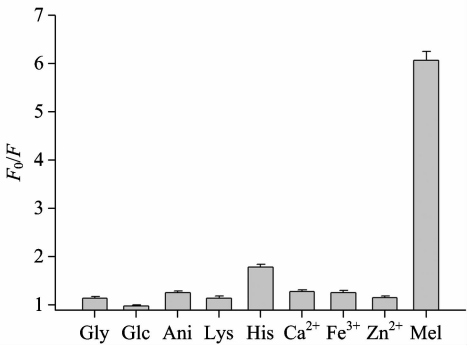

Fig. 5　The selectivity of the proposed method towards melamine detection. The concentration of melamine and other interferences are 150 μmol · L^{-1}

Table 2　Determination of melamine in spiked milk sample

Spiked milk sample	Mel added /(μmol · L^{-1})	Mel found /(μmol · L^{-1})	Recovery /%
1	0	0	—
2	50.0	47.5	95.0
3	60.0	59.8	99.7
4	70.0	69.7	99.6
5	90.0	90.4	100.4

3　Conclusions

In conclusion, a rapid, simple and practical fluorescent method for melamine detection was developed. This mehod relies on the inhibition effect of melamine on the formation of CuNPs through the coordination of melamine with Cu^{2+}. The as-prepared sensor exhibited high sensitivity and good selectivity for melamine, which can be applied to analysis of melamine in real samples with satisfactory results.

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三聚氰胺-铜(Ⅱ)配合物抑制 AT-双链铜纳米颗粒合成荧光检测三聚氰胺

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摘 要 基于三聚氰胺与铜离子配位反应并抑制 AT-双链铜纳米颗粒合成，构建了一种新型的“turn-off”策略检测三聚氰胺。当三聚氰胺存在时，与铜离子发生配位反应，使得后期合成铜纳米颗粒的铜离子浓度不够，导致铜纳米颗粒荧光减弱。在最优化实验条件下，对三聚氰胺检测的线性范围为 1～150 μmol·L^{-1}，检出限达 0.5 μmol·L^{-1}。此外，该方法还可以检测牛奶样品中的三聚氰胺，回收率良好。

关键词 铜纳米颗粒；铜-三聚氰胺配合物；非标记传感器；三聚氰胺检测

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