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Research Article

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Turn-off Fluorescence Quenching Based Determination of Melamine in Milk Using Unmodified Copper Nanoclusters

V.J. Shiva Swaraj[1], Gaurang Jain[1], Astha Jangid[1], Ansh Soni[1], Rajat Singh[2] and Naveen Kumar[1*]

[1]Amity Institute of Biotechnology, Amity University Rajasthan, Jaipur, India
[2]College of Horticulture and Forestry, Thunag, Mandi, Himachal Pradesh, India

***Correspondence to:**
Naveen Kumar
Amity Institute of Biotechnology,
Amity University Rajasthan,
Jaipur, India.
E-mail: nkft87@gmail.com

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Abstract

We report a highly cost effective, simple to use, environment friendly and highly sensitive assay for detection of melamine with L-ascorbic acid stabilized copper nanoclusters (CUNCs). Melamine, a well-known nitrogen rich compound, commonly used as adulterant in milk, has a positive charge and interacts with the highly fluorescent CUNCs through hydrogen bonding, which causes the aggregation of CUNCs following which there is a significant quenching of fluorescence that helps determine the presence of melamine in samples with concentrations as low as 0.01 μM which is 2000 times lower than that allowed by United States Food and Drug Administration (USFDA), which is 20 μM. Excellent results were obtained on testing real samples like milk, powdered milk and whey protein powder which were spiked with melamine, using the reported method. The limit of detection (LOD) established (0.01 μM), to the best of the knowledge, is the lowest reported using nanotechnology. This highly sensitive CUNCs-based fluorescence quenching method can find application in detection of melamine in milk and milk-based product samples.

Keywords

Melamine, Copper nanoclusters, Fluorescence quenching, Fluorescent nanoclusters

Introduction

1,3,5-triazine-2,4,6-triamine, more commonly known as melamine (chemical formula $C_3H_6N_6$) is an organic compound with 66% nitrogen content [1] and is widely used for production of resins and plastics. Due to this nitrogen content, melamine is commonly used to adulterate food products like milk, milk powder, infant formulas, protein supplements, pet food etc., which are valued for their protein content for unethical profits by few manufacturers. Cattle feed in China were added with melamine as a source of non-protein nitrogen from 1958 to 1978 [2]. Melamine forms insoluble crystals in combination with cyanuric acid which is known to cause kidney stones, leading to kidney failure and death [3]. Melamine caused a crisis in the period of 2006 to 2008 in US and China where large number of pets and infants were diagnosed with kidney problems, including kidney failure and death [1-3].

Due to the inability of Kjeldhal or Dumas methods in identifying nitrogen source (melamine) [4], a lot of work has been reported post 2007 crisis, for the detection of melamine using various methods like, gas chromatography/mass spectrometry [5], high performance liquid chromatography [6], capillary and moving reaction boundary electrophoresis [7, 8], chemiluminescence system [9], optical biosensor-based immunoassay [10, 11], aptamer based colorimetric detection [12], and many more.

Out of all the listed technologies, nanotechnology stands out when it comes to detection of melamine as an analyte. Gold nanoparticles [13] and silver nanoparticles [14] have been used successfully for developing colorimetric sensors. Moreover, over the years, ultra-sensitive 'turn-on' fluorescent sensors have been developed using carbon nanodots [15], gold nanoclusters (AuNCs) [16] and CUNCs [17]. A more interesting concept is of the fluorescence quenching method, where AuNCs [18] and triton X-114 [19] were used as fluorophores and the quenching of their fluorescence was utilized successfully for detection of melamine in samples with concentrations as low as 32 nM.

To the best of the knowledge, this is the first report of utilization of CUNCs for the detection of melamine based on 'turn-off' fluorescence quenching method, where samples with concentration of 0.01 µM were detected successfully which is 2000 times lower than the safety limit of 20 µM defined by USFDA and the European Union.

The reported method is highly sensitive, cost effective, and environment friendly compared to the other reported methods and is also easy to apply in real samples.

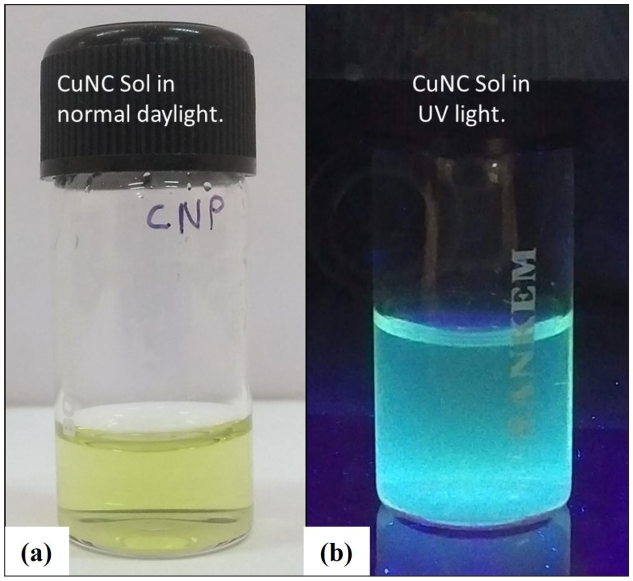


Figure 1: (a) CuNCs solution as observed in daylight and **(b)** CuNCs solution as observed under UV light.

Materials and Methods

Chemicals

Melamine (99%) was purchased from Sigma-Aldrich (St Louis, MO, US). Cupric chloride ($CuCl_2.2H_2O$) was purchased from Thermo-Fisher Scientific India Pvt. Ltd. (Mumbai, India). L-ascorbic acid, trichloroacetic acid and sodium hydroxide (NaOH) were purchased from Merck Ltd. (Mumbai, India). All the listed chemicals were of analytical grade and were used without any further purification. Millipore milli-Q (18 MΩ) water was used throughout the experiment. The milk and milk powder samples were sourced from the university's departmental store and whey protein powder was purchased from Dymatize Enterprises (LLC Dallas, TX, US).

Synthesis of CUNCs

The aqueous solution of L-ascorbic acid stabilized nanoclusters was synthesized by modifying a previously reported method of one pot 'green' synthesis method [20] 50 ml of 0.01 M cupric chloride was prepared and sonicated for 10 min, after which it was heated on a hot plate cum magnetic stirrer at exactly 85 °C with rigorous stirring. The temperature was monitored throughout the experiment using a Sigma handheld IR thermometer. After the temperature was constant, 8 ml of 0.25 M L-ascorbic acid was added dropwise post which the color of the solution changed to pale greenish yellow. As soon as the color change was observed, the heating was stopped, and the stirring continued for 12 h. The obtained CUNCs were light green in color when observed from the naked eye and exhibit blue fluorescence when exposed to ultraviolet (UV) light as seen in figure 1.

Sample extraction and preparation

The sample preparation method was adopted from [16], without any modifications, where 5 ml of milk was taken in a 15 ml centrifuge tube to which 1.5 ml of 2 M trichloroacetic acid was added. The tube was then sonicated for 10 min following which it was centrifuged at 13000 rpm for 10 min. The highly acidic supernatant was collected, and its pH was set to 7.0 with the addition of 1 M NaOH after which the supernatant was filtered with a 0.2 µm filter and diluted 25 times.

The milk powder and whey protein powder were reconstituted with water as directed on the product and the above stated method was applied for sample preparation. Stock solution of melamine with concentration 50 mM was prepared and used accordingly.

Instrumentation

Atomic forced microscopy (AFM) was executed using Bruker multimode scanning probe microscope, all fluorescence data was recorded using Perkin Elmer LS55 fluorescence spectrometer, Fourier transform infrared (FTIR) spectra spectra was obtained using Perkin Elmer FTIR-SPECTRUM 2, all UV visual spectral data were recorded using Thermo Scientific Multiscan GO.

Results and Discussion

Characterization of CUNCs

The structural properties given in figure 2a shows the AFM images of the as synthesized CUNCs which are uniformly dispersed, and their average size is approximately 65 nm and the 3-D illustration of the AFM data in figure 2b describes the maximum height of the particles to be 14.4 nm. The optical properties observed by fluorescence spectroscopy, as seen in figure 3, show the emission spectra distinctly at 455 nm with excitation wavelength being 386 nm. The stock shift ($\lambda_{emission} - \lambda_{excitation}$) observed is 69 nm which prove the particles have highly fluorescent property. The increase in quenching of fluorescence with increasing concentration of melamine can be observed in the graph depicted in figure 3, the same can be observed in figure 4 which shows the decrease in fluorescence when observed in a UV transilluminator.

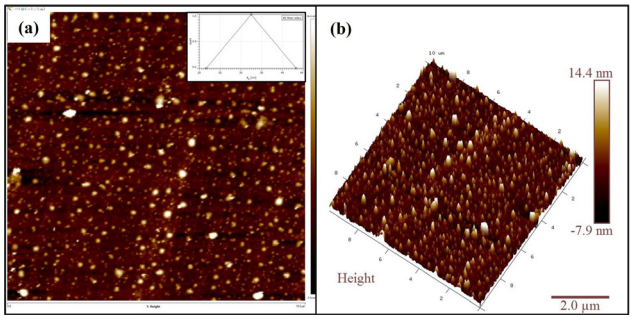


Figure 2: (a) The AFM image shows the uniformly dispersed as prepared CUNCs with insert graph of average radius being 32.4 nm and **(b)** The 3-D image shows the maximum height of the prepared CUNCs surface to be 14.4 nm.

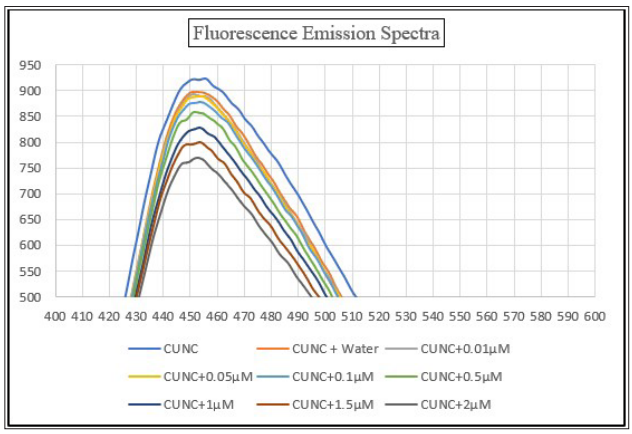


Figure 3: Fluorescence emission spectra for different concentrations of melamine mixed with fluorescent nanoclusters, where excitation wavelength = 386 nm.

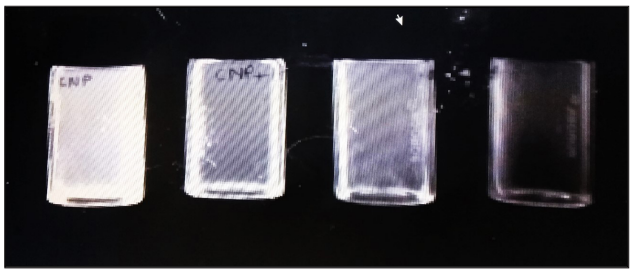


Figure 4: UV-transilluminator image depicting the decreasing value of fluorescence with increasing concentration of melamine from left to right (Left most being as prepared CUNCs, followed by CuNCs with 1.5 µM melamine, CuNCs with 2 µM melamine, and on the right most CuNCs with 50 µM melamine).

Principle of melamine detection using CUNCs

A schematic representation of the principle of detection of melamine is given in figure 5. Upon addition of melamine, which has a strong positive charge compared to the negative charge possessed by the CUNCs, the melamine molecules induce electrostatic interaction and rapidly form hydrogen bonds induced by the strong NH_2 group, with the L-ascorbic acid stabilized nanoclusters which is observable in the FTIR spectra, a distinct peak can be observed at wavenumber 1648.60, due to which the aggregation of the particles takes place and the fluorescence is quenched. This aggregation can

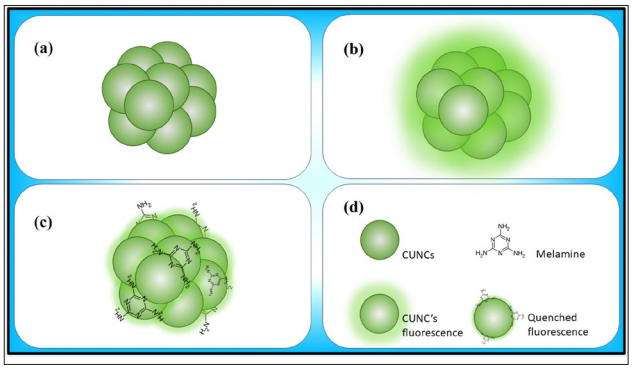


Figure 5: (a) Graphical representation of CUNCs, **(b)** Graphical representation of fluorescence emitting CUNCs upon excitation with UV, **(c)** Melamine molecules attaching to the nanoclusters through hydrogen bonding and causing the decrease in the fluorescence, and **(d)** Legend of scheme.

be observed in figure 6a where the AFM image clearly portrays the aggregation, and the cluster nature can be more distinctly observed. The maximum height of the particles upon addition of melamine also increases to 164.9 nm as seen in figure 6b, which ultimately reduces the fluorescence property of the CUNCs. With increasing concentration, the NH_2 induced aggregation results in an observable quenching as seen in figure 3 and figure 4. Using the Stern-Volmer equation, that is, I_0/I, where I_0 is the fluorescence of the as prepared nanocluster solution, and I is the fluorescence of the nanocluster solution upon addition of melamine. These values can be calculated, and the equation obtained using the standard curve y = 0.0736x + 1.0458, as seen in figure 7, can be used to calculate the concentration of melamine added, where 'x' denotes the concentration of melamine.

Optimization of assay conditions

The pH of the as prepared CUNCs aqueous solution was determined to be 2, and the concentration of the nanoclusters was estimated to be 8.125 x 10^{-8} g/mL. The reaction time for melamine sample in nanocluster solution was set to be 5 min for complete reaction.

The assay did not require any special optimization for reproduceable results, and the as prepared nanoclusters were used in all experiments. The prepared sample's pH was set to 7.0 for optimum stability of melamine.

Calibration curve and sensitivity

Known concentrations of melamine from 0.01, 0.05, 0.1, 0.5, 1, 1.5, 2 µM were added to CUNCs and observed their fluorescence spectra. The fluorescence intensity gradually decreased as the concentration of melamine increased (Figure 3 and figure 4). Figure 8 shows good linearity with concentration 0.01 - 2 µM with regression equation F_0/F = 0.0736x + 1.0485 (x = concentration of melamine) and the correlation coefficient value (R^2) = 0.994.

Analysis of melamine in pure and spiked real samples

Table 1 provides a comprehensive evaluation of the analytical parameters of the melamine detection method under the optimized assay conditions. For each sample type, we ex-

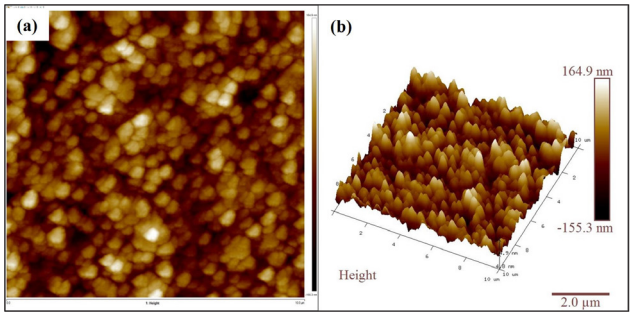


Figure 6: (a) The AFM image shows the aggregated CUNCs upon addition of melamine and **(b)** The 3-D image shows the maximum height of the CUNCs surface increased to 164.9 nm upon addition of melamine.

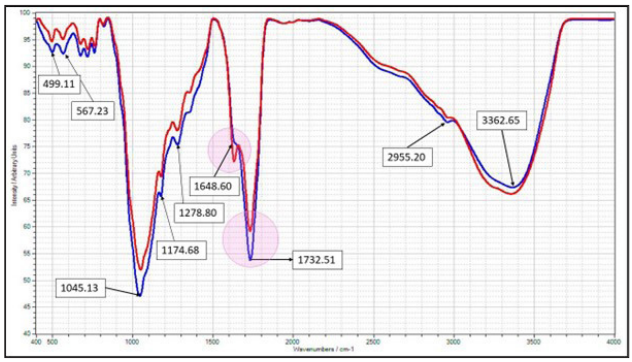


Figure 7: FTIR spectra depicting the NH_2 induced hydrogen bonding at the highlighted area. Blue line denotes the as prepared CUNCs, and the red line denotes the CUNCs with melamine.

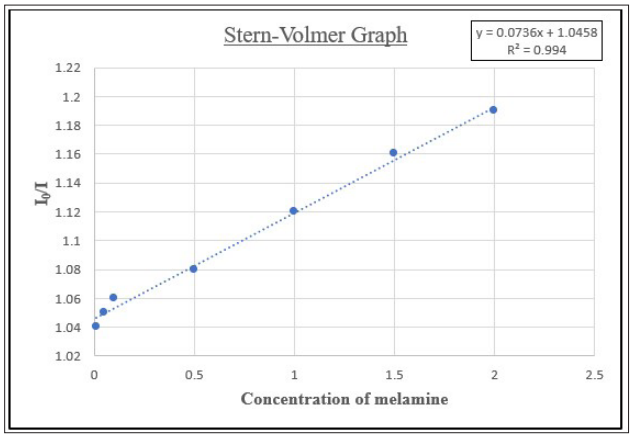


Figure 8: Graph for Stern-Volmer equation (I_0/I) with melamine concentrations: 0.01 µM, 0.05 µM, 0.1 µM, 0.5 µM, 1 µM, 1.5 µM, and 2 µM.

Table 1: Recovery study in spiked milk sample. Results are presented as mean ± standard deviation.

Sample	Melamine added (mcg/L)	Recovered (mcg/L)	Percentage of recovery	Percentage RSD
Milk	1.2	1.0 ± 2	83.33%	14.78%
	63	61.098 ± 6	96.98%	4.11%
Reconstituted milk	6.3	6.08 ± 3	96.50%	32.43%
	126	127.2 ± 8	100.95%	5.09%
Reconstituted whey protein supplement	12	11.26 ± 4	93.83%	19.23%
	189	191.01 ± 13	101.06%	4.47%

amined the recovery of melamine by comparing the added melamine concentration to the measured concentration. The results demonstrated the method's efficiency in recovering melamine, with recovery percentages ranging from 83.33% to 101.06%. The percentage of RSD values, which indicate the precision of the method, ranged from 4.11% to 32.43%, showcasing varying levels of precision for different sample conditions.

A comparative study of similar methods used for detection of melamine is given in table 2. It presents a concise overview of various fluorescence-based detection methods, categorized into "turn-on fluorescence enhancement" and "turn-off fluorescence quenching" approaches, for specific analytes. Each method is defined by its components or recognition elements, the linear range within which it operates effectively, and its LOD, representing its sensitivity. Notable examples include the "polythymine stabilized CuNCs" method with a linear range of 0.1 - 0.6 µM and an LOD of 0.095 µM, as well as the "ascorbic acid stabilized CuNCs" method from this study, offering an impressive linear range of 0.01 - 2.5 µM and an LOD ranging from 0.01 - 0.029 µM.

Interference study

To determine the specificity of the proposed method for detection of melamine, a set of possible interferents were studied where the change in fluorescence ratio was observed upon addition of the interferents with the CUNCs solution. The results concluded that the suspected interferants like glucose, urea, formalin, hydrogen peroxide, sucrose, dextrose, and calcium (2.5 µM) have very less selectivity to CUNCs when compared to melamine (0.5 µM) as depicted in figure 9.

Table 2: Comparison of other methods using fluorescence-based method of detection.

Fluorescence based method of detection	Components/recognition elements	Linear range	LOD	Ref.
Turn-on fluorescence enhancement	C-dots with Hg^{2+}	1 - 20 µM	0.3 µM	[15]
Turn-on fluorescence enhancement	BSA-AuNCs with Hg^{2+}	0.5 - 10 µM	0.15 µM	[16]
Turn-on fluorescence enhancement	Polythymine stabilized CuNCs	0.1 - 0.6 µM	0.095 µM	[17]
Turn-off fluorescence quenching	TCA and CPE with Triton X-114	1 - 6 mg/L^{-1}	800 µM	[19]
Turn-off fluorescence quenching	TPN-AuNCs	0.09 - 100 µM	0.032 µM	[18]
Turn-off fluorescence quenching	Ascorbic acid stabilized CuNCs	0.01 - 2.5 µM	0.01 - 0.029 µM	This work

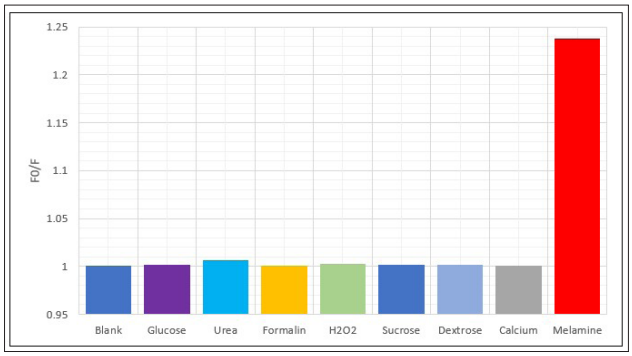


Figure 9: Interference study of common adulterants in milk compared to melamine, where the concentration of suspected interferents is 2.5 µM as compared to melamine 0.5 µM.

LOD

The LOD was calculated to be 0.01 µM, which is 2000 times lower that the lowest reported method to the best of the knowledge.

Conclusion

The study introduces a cost-effective, user-friendly, and highly sensitive nano-sensor for the detection of melamine in milk and milk products. This method harnesses the unique fluorescence quenching capability of CUNCs induced by hydrogen bonding-induced aggregation upon the addition of melamine. The work addresses the critical issue of melamine adulteration and offers a practical solution that is sensitive, cost-effective, and straightforward to use. This nanosensor represents a significant advancement in the field and holds great promise for enhancing the safety and quality control of milk and milk-based products, aligning with the ongoing efforts to combat melamine-related food adulteration.

Acknowledgements

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Conflict of Interest

None.

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