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Simple Visual Detection of Raw and Pasteurized Milk Adulteration with Melamine by Silver Nano Particles in Egypt

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Abstract: Much attention has been devoted to melamine (MA) adulteration analysis in food products in accordance with the food safety standards. As melamine *in vivo* can be hydrolyzed to cyanuric acid which in turn associates with melamine to form reticulations, resulting in the formation of kidney stones depending upon urine pH that might cause organs failure and even death in humans and animals. From the fact that milk is very essential feed stuff and daily used, so the purpose of this paper is to develop a sensitive and low-cost visual colorimetric method for detection of melamine using simple circuitry by using Nano silver technology. The melamine induces the aggregation of nanoparticles due to electron donor-acceptor interaction between melamine and p-nitroaniline at the Ag NPs interface, resulting in a shift in the surface plasmon band and a consequent color change of the Ag NPs from faint pink to red, as low as 0.1 ppm melamine in raw and pasteurized milk could be distinguished upon a color change of solution in few minutes.

Key words: Adultration · Milk · Silver Nanoparticeles · Melamine · Visual Detection

INTRODUCTION

Melamine is a kind of triazine analog with three amino groups, which is widely used in the early 1950s in plastic engineering and agriculture as an important industrial material. Melamine is forbidden to be used as a food related additive ingredients. Because of its high nitrogen content (66%) and low cost, so it used in adulteration of food products in order to increase apparent protein content when tested by the Kjeldahl and Dumas tests, which estimate food product protein levels by measuring the nitrogen content as it can be easily misled by adding nitrogen rich compounds such melamine, melamine was adulterated in protein-rich diets by unethical manufacturers was illegal [1]. Addition of 1% melamine in food causes protein content to artificially boost more than 4%. Melamine could not be metabolized in human body and could form insoluble complexes with cyanuric acid, depending on urine pH, which could lead to crystallization and subsequent tissue injury, in this way the excessive intake of melamine will result in the formation of insoluble melamine cyanurate crystal in kidney and finally cause renal failure [2, 3]. In early 2007, it became a topic of discussion when hundreds of pet death occurred due to pet food contamination with melamine [4]. Numerous cases of renal failure in children during September 2008 in China attributed to consumption of infant formulas were illegally adulterated by melamine that led to kidney stone [5-7].

Recently, silver nanoparticles (Ag NPs) and gold nanoparticles (Au NPs) have been widely used as colorimetric methods for chemical sensing and biosensing of various substances [8] such as viruses [9], protein [10], DNA [11], cancerous cells [12], metal ions [13-15], mercury [16], small molecules [17-19] and in detection of melamine substance in milk as a visual simple method [20]. Recently, the use of microorganisms to synthesize functional nanoparticles has been of great interest. Microorganisms can change the oxidation state of metals [21]. Metallic nanoparticles can be obtained by physical, chemical or biological methods. However, biological synthesis is reliable and eco-friendly that do not use toxic chemicals in the synthesis protocol and has received particular attention [22-24].

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The principle of silver nanoparticles (Ag NPs) in detection of melamine occur when the nanoparticles approach each other and aggregate, the color of the solution will change due to shift of the surface plasmon band to a longer wavelength. The reduced silver nanoparticles (Ag NPs) which are negatively-charged can attach with positively charged exocyclic amino groups (-NH2) in melamine, resulting in melamine molecules attached to the surface of Ag NPs and so the electrostatic force between silver nanoparticles (Ag NPs) and melamine counteracts the effects of Van der Waals' force between silver nanoparticles molecules, with the result of homodisperse of Ag NPs causing color change. A safety limit of melamine ingestion has been estimated at 2.5ppm for adult food and at 1 ppm for infant formula by the Food and Drug Administration (FAD) in USA [25]. The maximum residue level of melamine in infant formula is legally regulated at 1 ppm by Chinese government after the melamine accident [26]. Currently, several methods have been employed for the determination of melamine in milk and milk based products, such as gas chromatography (GC) [27], high performance liquid chromatography (HPLC) [28-30]. high performance liquid chromatography/mass spectrum (HPLC/MS) [31, 32], surface enhanced Raman spectroscopy [33], Capillary zone electrophoresis mass spectrum (CE/MS) [34] and immunoassay analysis (ELISA) [35]. These methods have high sensitivity, but most of them are time-consuming due to the complicated preparation of sample and also require expensive instruments and high personal cost. The above-mentioned disadvantages limit the application of the existing methods. Therefore, developing a rapid, simple, convenient and sensitive method for the determination of melamine has become increasingly attractive and necessary and may be developed to do at home by mothers.

Raw milk was pretreated with trichloroacetic acid and chloroform to remove the protein and fat, the proposed method can be used for the detection of melamine in raw milk by monitoring with the naked eyes or UV-Vis spectroscopy at room temperature. The whole detection process could be completed within 5min. The detection limit for melamine is 2.3 mM (0.29 mg/L) by this method, which is well below the safety limit (2.5 ppm in USA and EU; 1 ppm for infant formula in China) of melamine ingestion [25] and which is comparable with or better than the detection limits of the other methods [36]. Besides, the proposed colorimetric method is more convenient than Kjeldahl and Dumas tests, thus, can be used for the rapid detection of melamine in raw milk Swarnali *et al*. [20]. So we aimed from this work to detect milk adultration by melamine using simple visual method which can be developed to be used by mothers at home.

MATERIALS AND METHODS

Chemicals: Chloroform (CHCl3), trichloroacetic acid and sodium hydroxide were purchased from Sigma Company, Egypt. Melamine (2, 4, 6-triamino-1,3, 5-triazine) was purchased from Sprea Misr for chemicals and plastic company. Double distilled de-ionized water was used in all experiments. All solvents and reagents were of analytical grade and were used without further purification.

Apparatus: SCILOGEX MS-H-Pro Magnetic stirrer from CE, HETTICH Centrifuge from UK, T60 UV-Visible spectrophotometer CE, JENWAY 3510 PH meter from UK, The (SEM)images were taken by using SEM Model Quanta 250 FEG (Field Emission Gun) attached with EDX Unit (Energy Dispersive X-ray Analyses), with accelerating voltage 30 K.V., magnification14x up to 1000000 and resolution for Gun.1n),in The Egyptian Mineral Resources Authority, Central Laboratories Sector and TEM images were taken using JEOL-JEM 1400 transmission electron microscope (TEM) in Faculty of Agriculture Research Park Cairo University, samples for the TEM studies were prepared by placing a drop of the aqueous suspension of particles on carbon-coated copper grids followed by solvent evaporation under vacuum.

Synthesis of silver nanoparticles (Ag NPs) using algal extract:

Rapid Synthesis of Ag NPs from Macro Algae: One gm of seaweed powder was extracted with water and filtered. 90 ml of 1 mM AgNO3 solution were added to 10 mL of algal extracts slowly with magnetic stirring for even coating of silver and subjected to heating at 30°C for 40 min for reduction of Ag+ ions. The reduction of pure Ag+ ions was monitored by color changing from bale yellow to faint pink. This green synthesis was the modified method followed by Devi and Valentin Bhimba [37].

UV–Vis Spectra Analysis of Silver Nanoparticles Formed by Algae: The reduction of silver ions Ag + in aqueous extracts of marine algae and the formation of Ag NPs was monitored by measuring the UV-Vis spectra. UV-Vis spectroscopy analysis of produced silver nanoparticles was carried out as a function of bio reduction time at a wave length of 100-700 on Ultra violet-Visible spectroscopy (T80+UV/VIS) at Genetic Engineering and Biotechnology Research Institute (GEBRI).

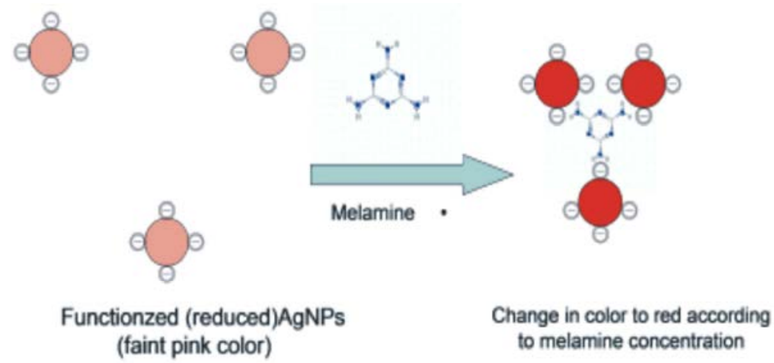

Fig. 1: Scheme of the principle for colorimetric melamine detection by the Ag Nps

In our experiments, when melamine was added into Ag NPs solution, the Ag NPs would aggregated together, simultaneously, the color of Ag NPs solution changed with spectral variations (Figure 1)

Colorimetric Detection of Melamine for Standard Curve: The colorimetric detection of melamine was studied using this functionalized Ag NPs solution at room temperature. To observe the effect of melamine on the functionalized AgNPs, 600 µl of melamine at concentration of (0.15,0.37,0.75,1.5,3.12,6.25,12.5,25,50,100 and 200 ppm) was added one at a time in 2 ml AgNPs solution and the resulting mixture was then allowed to stand for 2 minutes when the color changed from faint pink to reddish. The intensity of the color of the solution was found to increase with the increase of the melamine concentration.

Colorimetric Detection of Melamine in Raw Milk According to Swarnali *et al*. [20]: In order to detect any presence of melamine, we carried our experiment by taking thirty milk samples (4 from home, 14 from market, 4 from Vet. collage farm of Benha University and 8 from pasteurized milk). 4.0 ml of milk sample was diluted to make it 10 ml and 2.0 ml of 10% mixed solution of trichloroacetic acid and chloroform was added to it. The solution was then mixed with a vortex for 1 min to deposit protein in the sample matrix. Then the mixture was centrifuged at 10000 rpm for 10 min to separate the deposit. The supernatant was transferred into another centrifuge tube and adjusted to pH 8 with sodium hydroxide solution. The solution was centrifuged again and the final supernatant solution was used for melamine detection. To the 600 µl portion of the obtained solution, 2ml of functionalized AgNPs was added one at a time and the UV-Visible absorption spectra of the solution was taken after 2 minutes.

RESULTS AND DISCUSSION

In this study we detected milk adulteration with melamine as it low protein cost [1]. and has dangerous renal effect specially in children who may intake milk daily so we must detect it by rapid simple visible technique and developed it be available at home.

Our results concluded that we must characterize AgNPs to sure it in nanoparticle size so we must characterize Silver Nanoparticles by:

UV-Vis spectroscopy: The reduction of silver nitrate to AgNPs using aqueous algae extracts was monitored by measuring the UV-Visible spectrum. UV-Vis spectroscopy of AgNPs was characterized by one of the most widely used techniques of Jain and Pradeep [38]. The broadness of the peak is a good indicator of the size of the nanoparticle. As the particle size increases, the peak becomes narrower with a decreased band width and increased band intensity [39]. After 48 hrs of synthesis a broad peak was observed at 400- 450 nm belong to Ulva fasciata (Figure 2), which correspond to plasmon excitation of metal nanoparticles indicating the presence of silver nanoparticles. After initial reaction synthesis the band was at low wavelength and the reaction was carried out hasty. After 48hrs of reaction synthesis the band positioned at high wavelength due to aggregation of nanoparticles forming large size of nanoparticles that needed less energy and hence longer wavelength which indicated to poly dispersion of the nanoparticles [40]. So the reaction rate is directly proportional to reaction time till 48hrs of synthesis because after 48hrs the activity of AgNPs in the solution was stable at a period of 2 months [41]. Several researchers have observed absorption of a broad peak of colloidal silver in solution between 400 and 450 nm, which is assigned to surface plasmon excitation

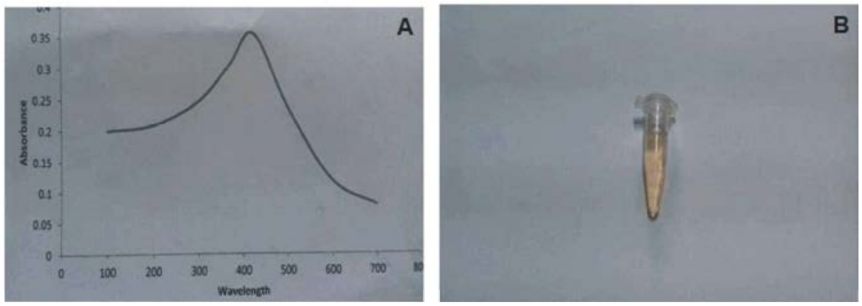

Fig. 2: A-B (A) UV-Visible Spectrum of funcationallzed AgNPs Solution and (B) the digital photographic image of the Solution

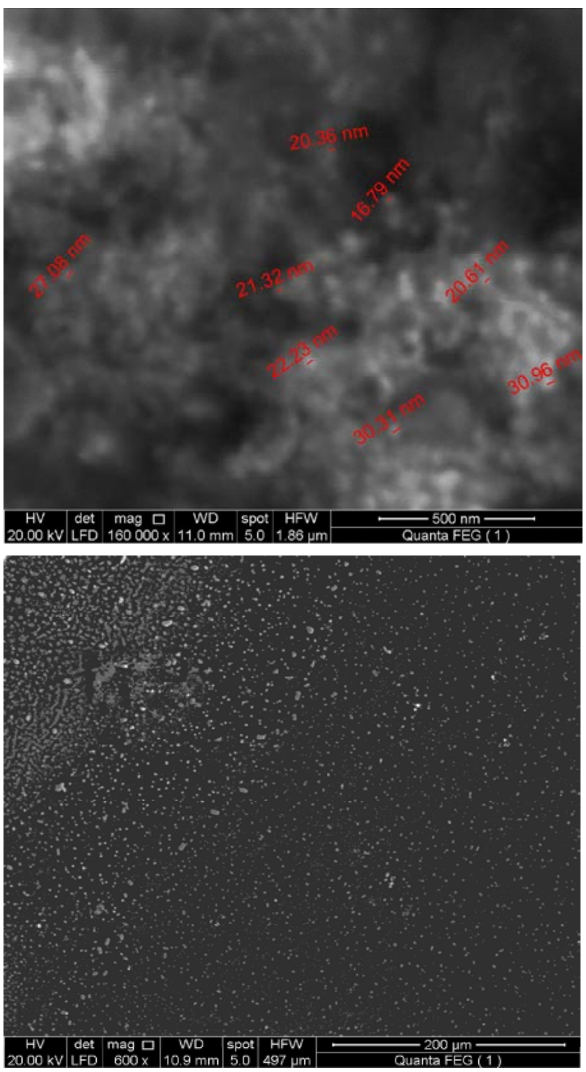

Fig. 3: Scanning Electron Microscopic images of silver nanoparticles biosynthesized by Ulva fasciata.

of the metal nanoparticles [42]. The fluorescence spectrum showed a broad emission peak of silver nanoparticles at 414 nm [43](Figure 2).

Scanning Electron Microscope: Our results obtained with scanning electron microscopy (SEM) showed that the silver nanoparticles have a uniform spherical shaped, well distributed in solution and a size range varying between 10-40 nm (Figure 3). Rajesh kumar *et al*. [44] reported that silver nanoparticles biosynthesized by algae with the spherical shape and average size of the nanoparticle was 96 nm.

Transmission Electron Microscope: Our results obtained from TEM micrograph recorded that the silver nanoparticles deposited on carbon coated copper TEM grid was shown in (Figure 4). This micrograph showed spherical AgNPs with a size range of 8-30nm in size, which agree with Roy *et al.* [45] found that silver nanoparticles synthesized by grape fruit extract were observed to have an average mean size of 19-23 nm and Devi and Valentin Bhimba [37] recorded that silver nanoparticles biosynthesized using Ulva lactuca showed low density dispersion and are in the range of 20-56nm in size.

Our results revealed that the morphology of AgNPs formed by Ulva fasciata using TEM and SEM showed a spherical crystalline shaped and it is known that the electronic and optic properties of metal nanoparticles are depended on their physical shape [46]. Also TEM and SEM showed that the size range is 8-40 nm. The size has relative advantages since particles are less than 100nm [37].

Melamine induces the aggregation of functionalized AgNPs due to electron donor-acceptor interaction between melamine and p-nitroaniline at the Ag NPs interface, resulting in a shift in the surface plasmon band and a consequent color change of the Ag NPs from faint pink to red. Based on this fact a visual detection of melamine by naked eye is possible. The color change was monitored using UV-VIS spectrophotometry.

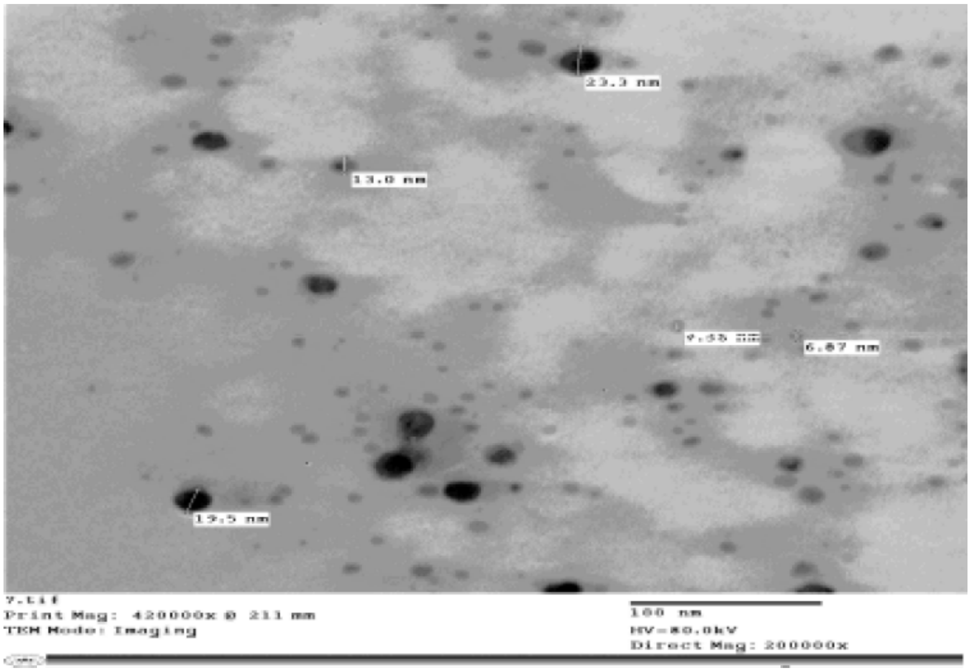

Fig. 4: Transmission Electron Microscopic images of silver nanoparticles biosynthesized by Ulva fasciata.

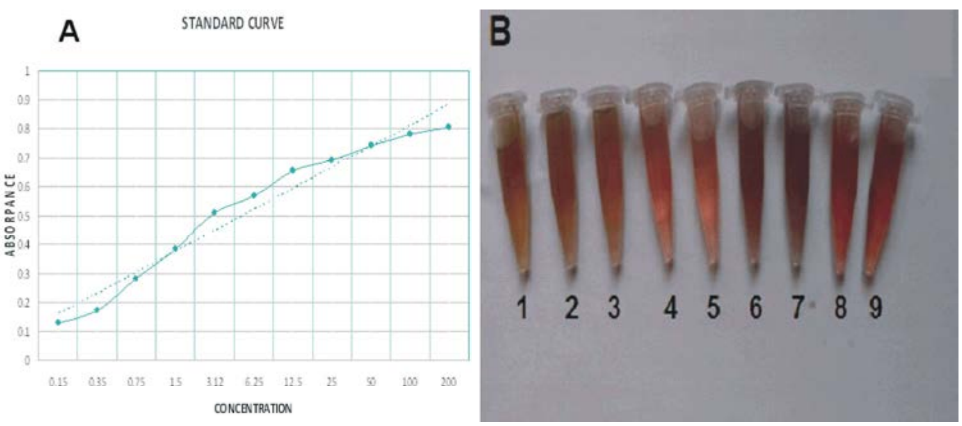

Fig. 5(A&B): B- standard curve of melamine. A-photographic different colors of different melamine concentrations.

After addition of melamine solution to the functionalized AgNPs solution, the color changed from faint pink to red (Figure 1), according to melamine concentration the change was observed in the UV-Visible spectrum, with the gradual increase of the concentration of melamine in the solution (Fig 5B). The absorption coefficient ratio of 625 nm and 415 nm (Ex 625 / 415) could be employed to measure the changes in absorption peak explicitly by the addition of different concentrations of melamine. The change in coefficient confirmed that the ratio increased with the increase of concentration of the melamine (Fig 5A, Firstly mentioned??). A calibration curve between the absorption co-efficient ratio and the concentration of melamine in the range of 0.15- 200 ppm showed a linear relationship (Figure, 5B). Thus this method would be useful for the estimation of melamine present in a sample quantitatively (Fig. 5A&B).

During the addition of melamine solution into the AgNPs solution, the pH of the solution was strictly maintained at 8 because melamine hydrolyses to form cyanuric acid at below pH 5 (pH<5) and above pH 8 (pH>10) [47]. The hydrolysed product, cyanuric acid is unable to induce aggregation of AgNPs [48].

Quantitative Analysis of Melamine in Raw Milk Samples: In our results the raw milk samples were treated as mentioned above. After treatment, 600 µl of the obtained solution was added to 2 ml of produced AgNPs at pH 8 and after 2minutes the UV-Visible absorption spectra of the solutions were taken. The colour changing from faint pink to red. The absorption co-efficient ratio (Ex625/415) for each milk sample was calculated for quantification and by comparing the absorption co-efficient ratio (Ex625/415) values of these samples with the values obtained from the calibration curve of known concentrations of melamine added by ppm (Figure. 5A), it was found that all milk samples were negative for presence of melamine, but the pasteurized milk samples contained 0.25-0.9 ppm of melamine, which is less than the safety limit assigned by various countries, as the safety limit of melamine ingestion has been estimated at 2.5ppm for adult food and at 1 ppm for infant formula by the Food and Drug Administration(FAD) in USA [25], which disagree with Swarnali *et al.*, [20] who reported that the milk samples available in market contain much greater amount than the safety limit assigned by various countries as it contain 180,200 and above 200 ppm.

CONCLUSIONS

Here we have reported that Ag NPs enables one to estimate quantitatively the amount of melamine adulteration in (raw and pasteurized) milk at ppm levels. The assay relies on the fact that melamine can induce aggregation of silver nanoparticles, so it is simple visual assay for detection of melamine by monitoring with the naked eye or a UV-Visible spectrophotometer without costly instruments and time consuming and can be developed to make test at home as milk is very essential feed stuff and is used daily to prevent causing kidney stones formation and organs failure and even death in humans and animals.

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