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Fluorescence "Turn-On" Detection of Melamine with Aggregation-Induced-Emission-Active Tetraphenylethene

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A few years ago, melamine was found as contaminant in pet food sold in the U.S. and Canada, as well as in milk, milk products, and infant formula, predominantly in China. Melamine has a number of industrial uses, but it is not approved for use in foods. In these incidents, however, melamine was illegally added to the foods at some point in the manufacturing process to boost the apparent protein content because of its high percent mass of nitrogen (66.6%), causing renal failure in pets, as well as in infants and children in China. The U.S. Food and Drug Administration (FDA) set a safe concentration level of melamine at 2.5 ppm for foods and at 1 ppm for powdered infant formula. Because there is an increasing public concern over food safety, selective detection methods for melamine in these products are highly desirable. Instrumental methods, such as gas chromatography mass spectrometry (GC-MS),[1] electrospray ionization mass spectrometry (ESI-MS),[2] high-performance liquid chromatography mass spectrometry (HPLC-MS),[3] and high-performance liquid chromatography (HPLC),[4] are available for the screening and quantification of melamine and its analogues in contamination events. These techniques are highly selective, but require expensive and complicated instruments that are not easy to operate. Recently, a colorimetric detection method by using Au nanoparticles has been reported, which could offer simple and sensitive detection of melamine.[5] Herein, we report the design and evaluation of alternative fluorescence "turn-on" detection of melamine in solution and on membrane. This system can sense the safe concentration level of melamine (1 ppm) in real milk products. The proposed method showed high precision and accuracy.[6] Thus, this fluorescence-based technique is suitable for routine and on-site use without using complicated instruments.

Recently, we[7] and others[8] have demonstrated that aggregation-induced emission (AIE)-active materials, first reported by Tang and co-workers,[9] have a potential utility in sensory fields.[10] AIE-active molecules show no emission in solution, but an intense emission when aggregated or in the solid state because of restriction of intramolecular rotations. Based on the state (aggregation)-dependent fluorescence, we designed an AIE-active tetraphenylethene (TPE) with cyanuric acid moieties, **1**, for the fluorescence sensing of melamine (Figure 1a). Because melamine/cyanuric acid combine together to form a stable adduct through multivalent hydrogen-bonding interactions (Figure 1b),[11] TPE integrated with cyanuric acid moieties **1** is seen to display an intense emission, that is, "turn-on" fluorescence, when it recognizes melamine and then forms aggregates (Figure 1c).

The TPE integrated with cyanuric acid **1** was synthesized by the reaction of 4-bromobutoxy-substituted TPE and cyanuric acid (Scheme S1 in the Supporting Information).

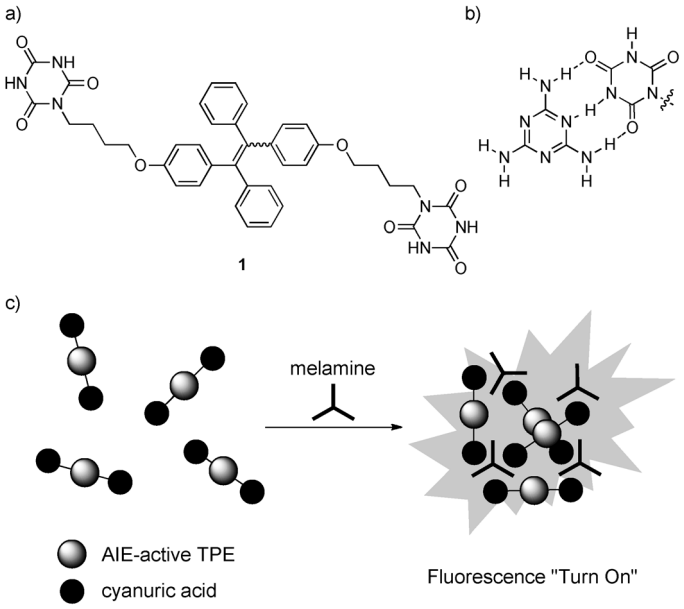

Figure 1. a) The chemical structure of **1**. b) Melamine–cyanuric acid adduct formed through multivalent hydrogen bonding. c) Schematic representation of a "turn-on" fluorescent sensing of melamine by using cyanuric acid modified tetraphenylethene (TPE) based on aggregation-induced emission (AIE).

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The sensory response of **1** to melamine was examined by monitoring the fluorescence spectral change upon addition of melamine ([**1**] = 10 μM). As shown in Figure 2, an acetonitrile solution of **1** was practically nonluminescent, with a

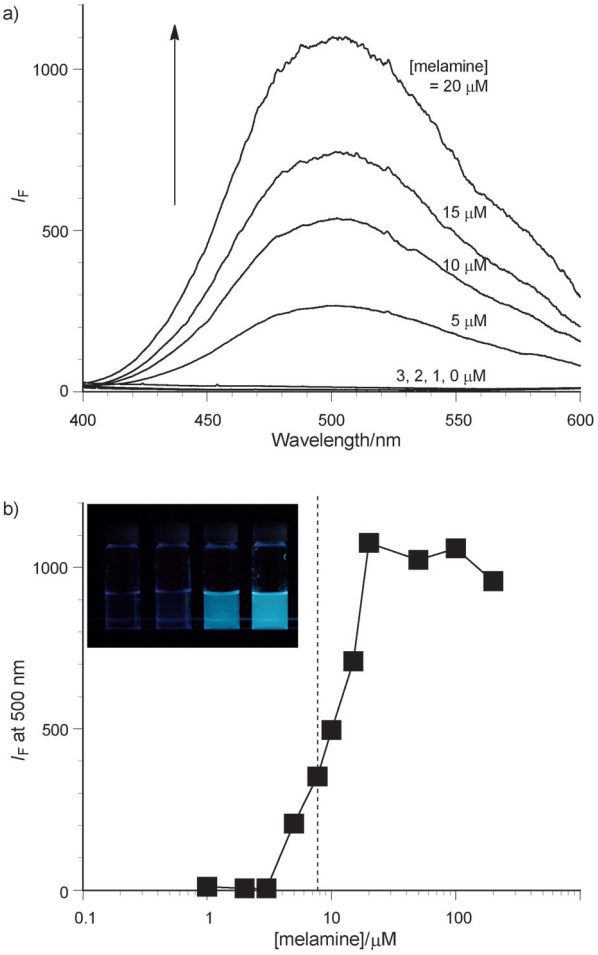

Figure 2. a) Fluorescence spectra and b) changes in fluorescence intensity (I_F) monitored at $\lambda = 500$ nm of **1** (10 μM) upon addition of melamine to a solution of **1** in acetonitrile ($\lambda_{Ex} = 350$ nm). The dotted line indicates the safe concentration level of melamine for infant formula (1 ppm). The inset shows photographs of **1** and the mixture with melamine (0, 1, 5, and 10 μM, from left to right) in an acetonitrile solution under irradiation with UV light at $\lambda = 365$ nm.

quantum yield (Φ_f) of 0.002 (relative to quinine sulfate as a standard). However, when melamine was added to the solution followed by ultrasonication, the mixture exhibited an intense emission. Importantly, we found that this system is well designed to identify melamine concentrations above 1 ppm (7.7 μM), which is the safe contamination level of melamine for infant formula set by the FDA. For example, at concentrations of melamine above 0.6 ppm (5 μM), the emission started to appear within a few hours and the intensity increased as a function of melamine concentration.[12] The emission was easily detectable by the naked eye, as can be seen in the inset in Figure 2b. Above 2.6 ppm (20 μM) of melamine, the fluorescence intensity reached a constant value with a greater than approximately 200-fold enhancement ($\Phi_f = 0.022$). However, melamine concentrations below 0.6 ppm (5 μM) did not cause an emission under this set of conditions. The assay is qualitative or semiqualitative. As shown in Figure 2b, the fluorescence intensity increased linearly with melamine concentration in the range 5–20 μM. The line obtained could be used as a calibration curve for quantification of the amount of melamine in the analyte. The fluorescence enhancement upon addition of melamine is achieved in terms of aggregation triggered by multivalent complexation of melamine with **1**, which in turn induces enhancement of the emission, as illustrated in Figure 1c. After filtration through a 0.45 μm pore size membrane, the residual solution showed no emission, indicating that aggregates formed (Figure S3 in the Supporting Information).

The selectivity of this assay was confirmed by screening its fluorescence response to relevant analytes (Figure S4 in the Supporting Information). Again, TPE **1** (10 μM) showed a fluorescence response in the presence of melamine (10 μM). No fluorescence signal increases were observed in the presence of ammeline, ammelide, uracil, cytosine, or thymine (100 μM). Thus, TPE **1** only responded to melamine, but not to the other compounds. This is because the cyanuric acid units on **1** specifically recognize melamine.

Most importantly, when tested in powdered infant formula spiked with melamine, the assay responded at concentrations of melamine above 1 ppm. As shown in Figure 3, the "turn-on" fluorescence was clearly observable by the naked eye (also. see Figure S5 in the Supporting Information).

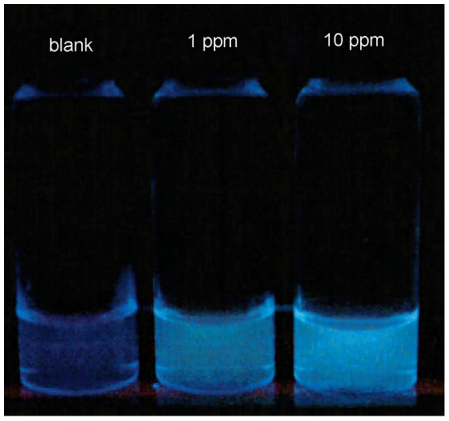

Figure 3. Photographs of **1** (10 μM) with extracts from melamine-contaminated infant powdered milk (blank, 1, and 10 ppm, from left to right) in an acetonitrile solution under irradiation with UV light at $\lambda = 365$ nm.

Next, with practical applications in mind, the membrane-based sensing of melamine was demonstrated. The membrane (or paper)-based sensors have attracted much attention for point-of-care testing alternative to conventional analytical instrumentations, because the devices are simple, portable, inexpensive, disposable, and use low sample volume.[13,14] In the sensing study, TPE **1** was adsorbed on hydrophobic poly(vinylidene difluoride) (PVDF) mem-

brane.[15] Acetone solutions of melamine at various concentrations (0, 1, 5, and 10 ppm) were spotted onto the test pieces of the membrane filter (6 mm in diameter).[16] When tested in powdered infant formula spiked with melamine, a blue emission was found within a few minutes at concentrations of melamine above 1 ppm, as show in Figure 4.[17] Thus, the test pieces detect the safe contamination level of melamine for infant formula set by the FDA by the naked eye.[18]

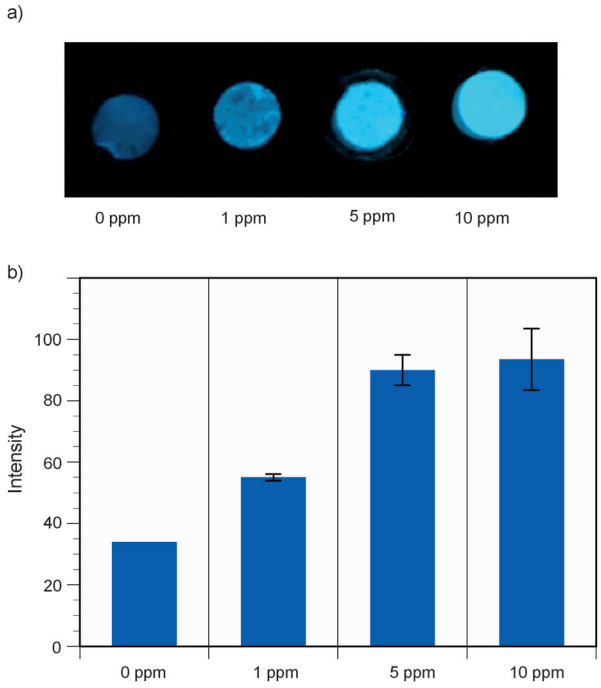

Figure 4. a) Images of test papers for the detection of melamine in infant powdered milk (blank, 1, 5, and 10 ppm, from left to right) with UV light at $\lambda = 365$ nm. b) The digital imaging of emission of the membrane by analyzing the digital photograph shown in a).

In conclusion, a "turn-on" fluorescence assay for detecting melamine has synthesized. The design takes advantage of the AIE of TPE to create a simple assay without using complicated instruments. Furthermore, this assay shows selectivity for melamine and also allows the detection at a safe concentration level in real powdered milk (1 ppm). Further study is currently in progress.

Experimental Section

Sensing studies of powdered infant milk with 1 in acetonitrile: A solution of powdered infant milk (40 mL, 5.4 g) in water was spiked with melamine (4.0 mg). This milk contained 1 ppm melamine. After ultrasonication for 30 min, 2 mL of the mixture was diluted with acetonitrile (78 mL). The sample was centrifuged at 3000 rpm for 30 min, and 2 mL of the supernatant was filtered with 0.45 μm pore size membrane. The filtrate was evaporated under reduced pressure followed by addition of acetonitrile (4.5 mL) and a stock solution of **1** (0.5 mL, 100 μM) to the resulting residue. After ultrasonication for 3 h, the sample was subjected to the fluorescence measurement ($\lambda_{Ex} = 350$ nm).

Sample preparation for imaging detection: A typical example is as follows (10 ppm). An ethanol solution of powdered infant milk (10 mL, 1.35 g) in water was spiked with melamine (0.10 mg). After addition of ethanol (5 mL) and stirring for 5 min, the mixture was filtered through cotton. The filtrate was used for the sensing study.

Imaging detection of melamine on membrane filter: An acetone solution (20 μL) of **1** (100 μM) was spotted on PVDF membrane with a diameter of 6 mm (0.28 cm^2) with a micropipet. After solvent evaporation, an acetone solution (20 μL) of melamine (0, 1, 5, and 10 ppm) was spotted on the membrane filter. Again, after solvent evaporation, spotting of the acetone solution of melamine (20 μL) on the membrane filter and solvent evaporation was replayed. After spotting of the melamine and solvent evaporation, process was repeated (total five times), one drop of ethanol was spotted to keep. Then, the membrane filter spotted with melamine was mounted on a slide glass to subject the sensing study under UV irradiation of black light.

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Keywords: aggregation · fluorescence · sensors · tetraphenylethenes

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[12] The response rate was dependent on the concentration of melamine and also the concentration of **1**. For example, a mixture of **1** (10 μM) and melamine (10 μM) showed an emission after sonication for 1 h, but a mixture of higher concentration of melamine ($\geq$15 μM) showed an emission within 30 min. On the other hand, 10 μM of **1** required 1.5 h to detect 7.7 μM (1 ppm) of melamine, but 20 μM of **1** detected it within 30 min. For details, see Figures S1 and S2 in the Supporting Information.

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[15] The membrane-based sensing studies were also examined by using paper filter, nitrocellulose membrane, nylon membrane, and silica-gel TLC.

[16] The response was dependent on the solvent used for spotting of melamine. For example, acetonitrile or ethanol was used, the test piece only detected at concentrations of melamine above 10 ppm.

[17] Under this set of conditions, the membrane was wet with ethanol during detection. When the membrane was dried off, a blue emission was observed despite the absence of melamine. Then, the control experiment (0 ppm) was usually required.

[18] The procedure for our assay is complicated at the present stage. To simplify procedures for the sensing, further study is required.

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