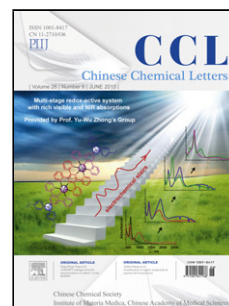# Accepted Manuscript

Communication

Visual recognition of melamine in milk *via* selective metallo-hydrogel formation

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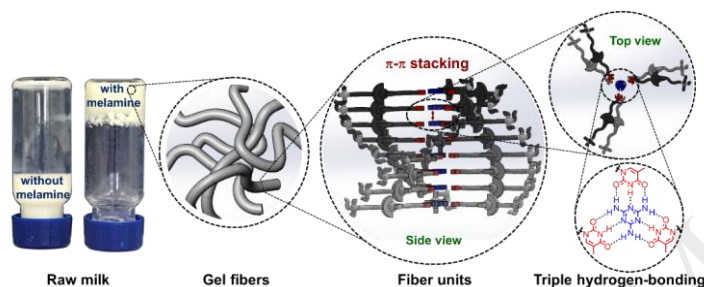
Graphical abstract

Raw milk   Gel fibers   Fiber units   Triple hydrogen-bonding

A novel protocol allowing convenient and highly selective visual recognition of melamine in raw milk *via* selective metallo-hydrogel formation at a concentration as low as 10 ppm without any tedious pretreatment has been developed.

ABSTRACT

A series of novel six-coordinated terpyridine zinc complexes, containing ammonium salts and thymine fragment at the two terminals, have been designed and synthesized, which can function as highly sensitive visualized sensors for melamine detection via *selective metallo-hydrogel formation. After fully characterization by various techniques, the complementary triple-hydrogen-bonding between the thymine fragment and melamine, as well as π-π stacking interactions may be responsible for the selective metallo-hydrogel formation. In light of the possible interference aroused by milk ingredients (portions, peptides and amino acids) and legal/illegal additives (urine, sugars and vitamins), a series of control experiments are therefore involved. To our delight, this visual recognition is highly selective, no gelation was observed with the selected milk ingredients or additives. Remarkably, this new developed protocol enables convenient and highly selective visual recognition of melamine at a concentration as low as 10 ppm in raw milk without any tedious pretreatment.*

Keywords:
Hydrogen-bonding interaction
Pincer zinc complex
Melamine
Metallo-hydrogel
Visual recognition

In recent decades, supramolecular gels have gained considerable attentions due to their diverse applications in the fields ranging from chemistry, physics, biology to material sciences [1-5]. Among them, the less-studied molecular metallogels are considered as one of the most fascinating "smart" soft materials [6,7], probably due to integrating the specific characteristics of metals into the gels. Along with other non-covalent interactions (such as van der Waals, π-π stacking and hydrogen bonds interactions), incorporation of metal provides subtle control of molecular assembly, gel morphology and stimuli responsive properties by tuning the metal-metal interactions [8-11]. Furthermore, suitable guest molecules might also assist or hinder these weak interactions to benefit metallogels formation or collapse, which also provides a new plausible approach for visual recognition of the guest molecules [12-14].

Due to its high nitrogen content (66%) and low price, melamine has been selected and illegally added to increase the protein reading level of dairy products [15-17]. However, melamine can induce renal disorder and even death, especially, for infants [18-21]. Consequently, a variety of expensive instruments, including liquid chromatography-mass spectrometry (LC-MS) [22], gas chromatography-mass spectrometry (GC-MS) [23], low-temperature plasma-mass spectrometry (LTP-MS) [24], surface-enhanced Raman scattering (SERS) [25], and enzyme-linked immunosorbent assay (ELISA) [26], have been applied for melamine detection. Although high sensitivity, high accuracy and low detection limits are usually observed, the detectable samples have to be tediously pre-processed and extracted before the analysis by using these expensive and complicated instruments [27-29], which is hardly suitable for the real-time and on-site direct detection of melamine in dairy products, especially for non-specialists.

In consideration of the reversibility, specificity, directionality of hydrogen bonding between melamine and thymine or its derivatives [30], such complementary triple N-H···O and N-H···N interactions have been regarded as a key issue for several reported melamine detection *via* molecular assembly [31]. However, to the best our knowledge, there is only one example on visual detection of melamine *via* molecular gel formation with peptide-based cyanuric acid derivative [32]. No metallo-hydrogel formation has been reported for this purpose. Following our recent research interests in metallogel fabrication, characterization and their potential application in visual discrimination of isomers [33], homologues [34], and bioactive molecules [35], we would like to extend the study to develop a simple, straightforward and on-site protocol for melamine detection of dairy samples *via* selective metallo-hydrogel formation.

In order to realize our hypothesis, a series of novel unsymmetrical pincer zinc complexes **1a** and **1b** (Scheme 1), containing a thymine fragment and a quaternary ammonium tail with different length of linkers, were therefore designed and synthesized. In light of the low solubility of pincer zinc complexes in water, the ammonium tail was introduced to increase the complex solubility. The synthetic route was described in Scheme 1. By condensation of 1-(pyridin-2-yl)ethanone, 4-hydroxyl-benzaldehyde and aqueous ammonium in ethanol under basic conditions, teypridine **2** was synthesized, which is readily converted to terpyridine-substituted alkyl bromides **3a** and **3b** with different alkyl lengths (n = 3 and 6). After amination reaction with thymine or triethylamine, thymine derivatives (**4a** and **4b**) and quaternary ammonium bromides (**5a** and **5b**) were then obtained. After coordination of zinc acetate, quaternary ammonium bromides **5a** and **5b** were readily converted into terpyridine zinc complexes **6a** and **6b** in methanol at room temperature. Finally, by simply mixing thymine derivatives **4a** and **4b** and terpyridine zinc complexes **6a** and **6b** in DMF, unsymmetric pincer zinc complexes **1a** and **1b** with two different terminals were obtained, which could be further purified by simply crystallization from DMF and ethyl acetate.
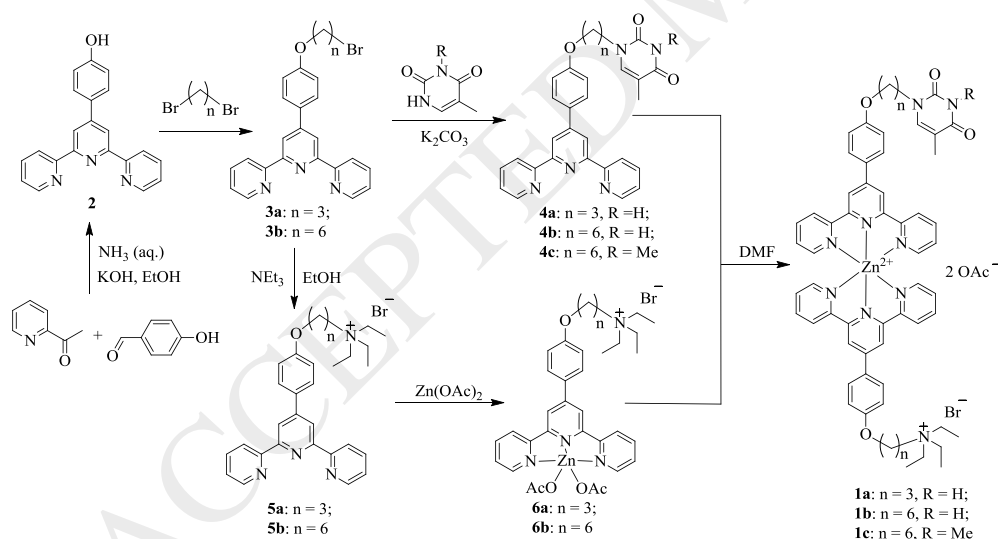
Scheme 1. Synthesis of unsymmetrical pincer zinc complexes **1a-c**.

Unsymmetrical pincer zinc complexes **1a** and **1b** are soluble in water at relatively high concentration. For example, 60 mg **1b** is readily dissolved in 1 mL water under ambient conditions (6 wt%), higher amount of **1b** could also be dissolved by gentle heating. To our delight, when a drop of saturated aqueous solution of melamine was added into 1 wt% of **1b** aqueous solution, after heating and cooling, a transparent metallo-hydrogel was readily formed, which may be considered as a new approach for visual discrimination of melamine *via* metallhydorgel formation. Subsequently, a series of gelation tests with different ratios of complex **1b** and melamine (from 3:1 to 1:3) at different concentration (1-6 wt%) were carried out (Table 1). Pleasingly, even at low gelation concentrations (0.5-1 wt%), gel formation could be still observed in some cases, though the gel was relatively weak (WG). The gel-sol phase-transition temperature (T_g) was determined by the "dropping-ball method" (Table 1). Higher stability and T_g were observed with the gel containing more amount of pincer complex **1b** and melamine. Unexpectedly, the higher amount of melamine induced higher thermostability and T_g. Similar outcomes were

also observed for pincer complex **1a**, with slightly worse stability of the corresponding metallo-hydrogels, probably due to the shorter alkyl chains. Although no gel formation was observed with normal organic solvents, a broad range of alcoholic solvents are suitable for gelation, and the resulting metallogels exhibited higher T_g values than those in water (Table S1 in Supporting information).

Table 1
Gelation test with pincer complex **1b** and melamine (**M**) in different concentrations and ratios in water.[a]

Entry	**1b:M**	Concentration (wt%) and T_g (°C)[b]					
		0.5	1	2	3	4	5
1	3:1	S/-	WG/-	G/44	G/49	G/51	G/52
2	2:1	S/-	WG/-	G/50	G/55	G/56	G/58
3	1:1	S/-	WG/-	G/53	G/59	G/62	G/64
4	1:2	WG/-	WG/-	G/56	G/62	G/64	G/ 65
5	1:3	WG/-	WG/-	G/61	G/70	G/72	G/74

[a] G, WG, and S denote gel, weak gel, and solution, respectively.
[b] Phase-transition temperatures determined by the "dropping-ball method".

The morphologies of xerogel networks obtained from complex **1a** or **1b** and melamine with the corresponding solvents were characterized by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Typical SEM images are presented in Figs. 1a and b. At 2 wt% and 1: 3 ratio of **1a** and **M**, wavy-hair—like fibers with approximately 2–5 μm long and up to 30 nm wide were observed in the metallo-hydrogel **1a** (Fig. 1a). In contrast, the image of 3 wt% metallo-hydrogel **1b** at the same ratio was slightly different and revealed long (up to 10 μm) and thin (*ca.* 20 nm wide) fibers (Fig. 1b). The same morphologies were observed in the TEM images (Figs. 1c and d). These long and thin gel fibers were also found in the metallo-gels **1b**/1-butanol and **1b**/cyclohexanol (Figs. S44 and S47 in Supporting information). Plates morphologies were observed for the metallo-gels **1b** in 1-pentanol and cyclopentanol (Figs. S45 and S46 in Supporting information).
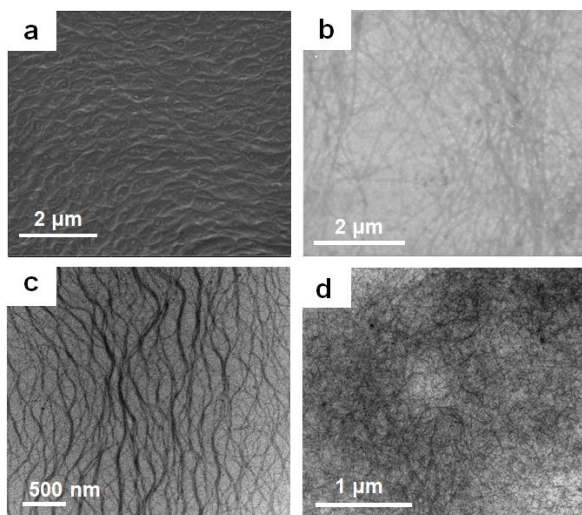

Fig. 1. SEM and TEM images of metallo-hydrogels of (a, c) **1a+M** (1: 3 at 2 wt%), and (b, d) **1b+M** (1:3 at 3 wt%).

In order to explore the possible driving forces for metallo-hydrogel formation and plausible mechanism of visual recognition, a series of experiments were carried out. Initially, temperature- dependent ^{1}H NMR spectroscopy studies of metallo-hydrogel **1b** in D$_2$O (1:3 at 2 wt%) were involved. To our surprise, in contrast to the ^{1}H NMR spectra of **1b** (Fig. 2a), there are less numbered but clearly broad signals in low intensity (Fig. 2b at 25 °C), indicating strong but complementary aggregation of pincer complex **1b** and melamine at the gel state under the ambient conditions. Upon warming to 75 °C by a step of 10 °C, the signals of the gel complex steadily sharpened. Compared with initial broad peaks at 25 °C, the N-H signal of thymine fragment (H_T) and O-H signal of CH$_2$ on the Et$_3$N$^+$C$_6$ chain (H_M) were steadily and obviously increased and downshifted (from 6.44 and 3.83 ppm to 7.11 and 4.45 ppm, respectively) along with the test temperature rising to 75 °C. These outcomes clearly indicated that the complementary triple hydrogen bonds between thymine fragment melamine were one of the main interactions responsible for the gelation process. Besides, the H$_\beta$ at the β-position of the middle pyridine was also downshifted (from 8.28 ppm to 8.72 ppm), which clearly suggested that π-π stacking between pyridines also contribute to the aggregation.
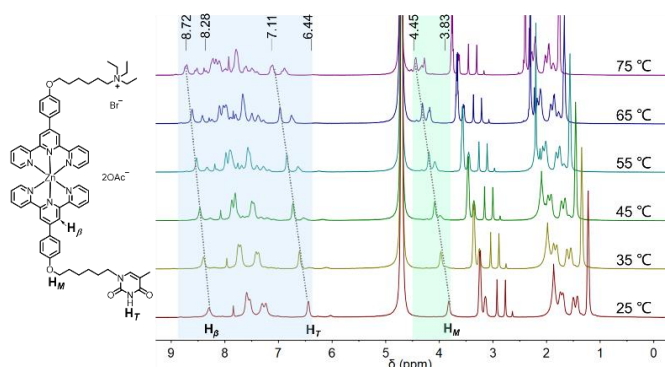
Fig. 2. Temperature-dependent ^{1}H NMR spectra of metallo-hydrogel formed by complex **1b** and melamine (1:3@2 wt%) in D$_2$O.

With these clues in our minds, control experiments involving pincer zinc complex **1c** (methyl protected analogue of **1b**, Scheme 1), triaminobenzene (a melamine analogue), the possible milk ingredients (like amino acids, illegal/legal additives including sugars, vitamins and urea) were carried out. When pincer complex **1c** or triaminobenzene was applied instead, under the standard gelation conditions (1:3 ratio at 2 wt%), no gelation was found in both cases (Fig. S60 in Supporting information). The results further confirmed that the importance of triple hydrogen bonds during assembly. Subsequently, when urea, lactose, glucose, glutamic, histidine and cysteine were used instead of melamine under the standard gelation conditions, no gelation was observed either (Fig. 3), though all these selected substances can form weak hydrogen bonds with thymine fragment. Hence, the visual recognition of melamine is highly selective *via* selective metallo-hydrogel formation.
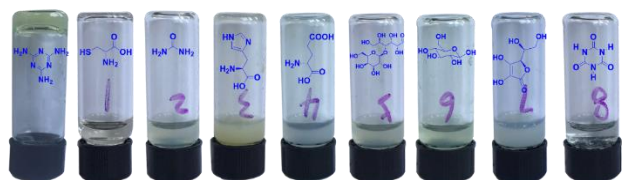

Fig. 3. Control experiments with various illegal/legal additives in milk for gelation test (6 mg additives with 3 wt% of complex **1b**).

We believe the direction and robustness of these complementary triple hydrogen bonds (Fig. 4c) will benefit molecular assembly to form a π-plate between melamine and thymine fragment, which can further yield fiber unit through π-π stacking interactions between the π-plates (Fig. 4b, side view). After twining, elongating and extending of the fiber units, long and thin fibers are therefore formed, which can trap the water molecules leading to formation of metallo-hydrogels (Fig. 4a).
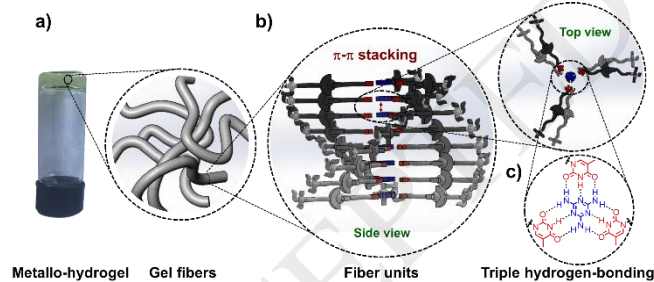
Fig. 4. Plausible assembly process of metallo-hydrogel prepared from pincer complex **1b** and melamine: a) metallo-hydrogel and gel fibers trapped water, b) fiber units formed *via* π-π stacking; and c) complementary triple hydrogen-bonding interactions.

Having the plausible aggregation and sensing mechanism in hand, we would like to test the visual recognition ability of the pincer complex **1b** to trace amount of melamine. After heating a vial containing 30 mg pincer complex **1b** in 0.5 mL water, no gel formation but a clear solution (A) was observed after heating and cooling process (Fig. 5a). When a drop of saturated aqueous melamine solution was added into the clear solution (A), a transparent gel was then formed after the standard heating–cooling process within 5 min (Fig. 5a). Subsequently, a series of aqueous melamine solution with different concentrations (1-1000 ppm) was added (0.5 mL) into a vial containing 30 mg pincer complex **1b** for visual discrimination. When the final concentration of melamine in the vial was greater than 10 ppm, gel formations were observed in all corresponding vials within a few minutes. Therefore, this protocol is promising for visual detection of melamine without usage of expensive and professional instruments.
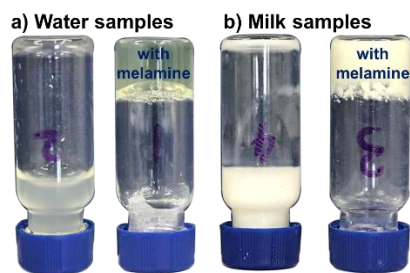
Fig. 5. Visual recognition of melamine by using a) a vial containing 6 wt% aqueous solution of pincer complex **1b** (solution A) with or without 10 ppm melamine in water, b) 6 wt% raw milk solution of pincer complex **1b** (solution B) with or without 10 ppm melamine.

Be aware of highly selective visual recognition of trace amount of melamine in aqueous samples and no impacts of milk ingredients, legal and illegal additives, we would like to investigate the possibility of using this protocol to detect trace amount of melamine added in raw milk without any pretreatment. To our delight, when 0.5 mL of the milk sample containing melamine (10 ppm) was added into the vial containing 30 mg pincer complex **1b**, a white turbid gel (6 wt%) was formed within 10 min after typical heating and cooling process (Fig. 5b). On the contrary, no gelation was observed for the milk sample without melamine under the otherwise identical reaction conditions (Fig. 5b). The gel formation is repeatable for several heating and cooling cycles, which further confirmed the visual discrimination ability of **1b** to melamine in raw milk. Compared with other known melamine detection approaches and even instrumental analysis involving GC-MS, HPLC-MS and LTP-MS, our newly developed visual recognition of melamine via selective metallo-hydrogel formation is much cheaper, straightforward and convenient. Even without any pretreatment, a sensitivity (10 ppm) comparable to instrumental analysis is achieved.

In conclusion, a cheap, straightforward and convenient approach for visual recognition of melamine *via* selective metallo-hydrogel formation has been established, even for raw milk samples without any pretreatment. The strong triple hydrogen-bonding interactions between melamine and thymine terminal of the unsymmetrical pincer zinc complex **1b** and additional π-π stacking may be responsible for the selective metallo-hydrogel formation, which have been confirmed by temperature-dependent ^{1}H NMR studies and related control experiments. Remarkably, even with raw milk samples, a comparable limit of detection to instrumental analysis is achieved, highlighting its potential application for quick practical on-site melamine-detection.

Acknowledgments

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