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Visualized detection of melamine in milk by supramolecular hydrogelations†

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We reported a visualized detection system for melamine based on supramolecular dydrogelations.

Melamine, with high nitrogen content (66%) and low cost, was illegally used in adulterating milk to boost the apparent protein level. However, melamine has a deleterious effect on human body and can lead to serious tissue injury in children,[1] such as acute kidney failure, urolithiasis, bladder cancer, and even death due to the formation of melamine–cyanuric acid crystals in the kidneys.[2] Therefore, it is of great importance to detect melamine in foods. For detecting melamine, various methods have been reported, including gas chromatography-mass spectrometry (GC-MS),[3] liquid chromatography-mass spectrometry (LC-MS),[4] enzyme-linked immunosorbent assay (ELISA),[5] and fluorescence spectroscopy,[6] *etc.* Although the existing methods can provide both accuracy and high sensitivity for melamine detection, all require expensive and complicated instruments. Moreover, the melamine is usually needed to be extracted from the milk to render its detection,[6c,7] thus making the on-site and real-time monitoring difficult. There is an urgent need to develop simple, rapid, easily accessible, and cost-effective methods to detect melamine in foods.

Supramolecular hydrogels are advantageous soft biomaterials because of their ease of synthesis, biocompatibility, degradability, and fast responses to external stimuli.[8] They have led to diverse applications such as tissue engineering,[9] drug delivery,[10] analyte detection,[11] *etc.* Recently, their application in sensing attracts extensive research interests. Supramolecular hydrogel-based sensors can not only provide an unambiguous visual change in the material physical properties but also, in contrast to colorimetric and fluorescent sensors, there is no need of any instruments and transporting samples to a laboratory. Such an advantage makes rapid, real-time and on-site monitoring possible. To date, many supramolecular hydrogel-based sensors have been developed for various important analytes, including enzymes,[11f,12] glucose,[13] nitric oxide[14] and metal ions,[15] *etc.* For instance, Xu and co-workers have reported on a simple assay for screening inhibitors of an enzyme (acid phosphatase) based on enzyme-triggered gel formation.[12a] Using a similar strategy, McNeil and coworkers also developed a modular system for screening protease activity *via* the sol–gel phase transition and for monitoring artificial blood clotting.[12b] Besides, Zhang and co-workers developed a simple visual biosensor for glucose detection based on the responsiveness of the peptide-based self-assembly to glucose metabolism.[13] Hamachi *et al.* reported a simple gel–sol phase transition catalyzed by H_2O_2 for the detection of several important enzymes.[11f] All these efforts advance the research progress of the supramolecular hydrogel-based detection systems. In this study, we reported on a simple and visual assay for the detection of melamine based on supramolecular hydrogelation.

It is well-known that melamine and cyanuric acid (CA) or its derivatives can form a stable complex through the interaction between diaminopyridine and diimide moieties, exhibiting three complementary NH–O and NH–N hydrogen bonds.[16] Such triple hydrogen bonding is considered to be particularly useful for controlling molecular self-assembly due to the reversibility, specificity, directionality, and cooperative strength of this class of interaction.[17] Inspired by this principle and the nature of the self-assembled peptide systems, we opted to design a peptide-based CA derivative with self-assembling properties for the detection of melamine. The derivative could self-assemble into nanofibers but not hydrogels due to the relatively weak inter-fiber interactions. With the assistance of the triple hydrogen bond from CA–melamine, the nanofibers might be cross-linked into 3D fiber networks, leading to the dramatic visualized phase

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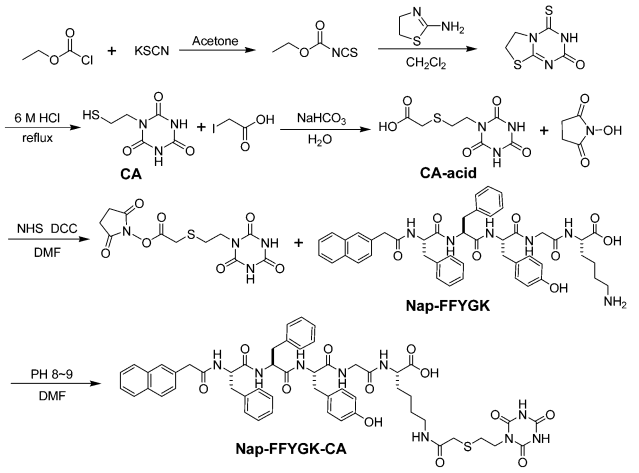

Scheme 1 The synthetic route to Nap-FFYGK-CA.

transition from a solution to a gel. This process could be developed into a simple, real-time, and on-site sensing method for melamine.

In order to test our hypothesis, we designed the molecule of Nap-FFYGK-CA (Scheme 1). Many peptide derivatives based on FF or FFY are molecules with excellent self-assembling properties.[18] We envisioned that our designed molecules might self-assemble into nanofibers with good water dispersity. The CA moiety was used to form a complex with melamine to increase the inter-fiber interactions for hydrogelation. The synthetic route was described in Scheme 1, we firstly prepared CA-acid in four steps with a total yield of about 70%. Nap-FFYGK was produced by standard solid phase peptide synthesis (SPPS). We then obtained Nap-FFYGK-CA by coupling CA-acid with the peptide. The pure compound was achieved by reverse phase high performance liquid chromatography (HPLC).

After obtaining Nap-FFYGK-CA, we tested its self-assembling properties by the heating–cooling process. We firstly prepared its phosphate buffer saline (PBS, pH = 7.4) solution at a concentration of 0.5 wt%. After a heating–cooling process, we observed the formation of a clear solution (Fig. 1A), suggesting that the compound itself could not form a hydrogel at this concentration. We observed a light beam when we used a laser pointer to shine the solution, indicating the presence of nanoscale materials in the solution. We then tested whether the addition of melamine would lead to hydrogelations or not. We mixed the solution of Nap-FFYGK-CA at a final concentration of 0.5 wt% and the solution of melamine with different concentrations. As shown in Fig. 2B, the solution changed to a hydrogel within 10 minutes when the concentration of melamine was higher than 35 ppm. We also observed the hydrogel formation when the concentration of melamine was 20 ppm, but the gelation took a longer time of about 8 hours. These observations clearly indicated the success of our design. As the sol–gel phase transition can be easily identified by the naked eye, this system might be suitable for the detection of melamine without instruments.

We also tested whether our method could be used to detect melamine in foods and biological fluids. We choose melamine tainted milk and urine because of the importance of detection

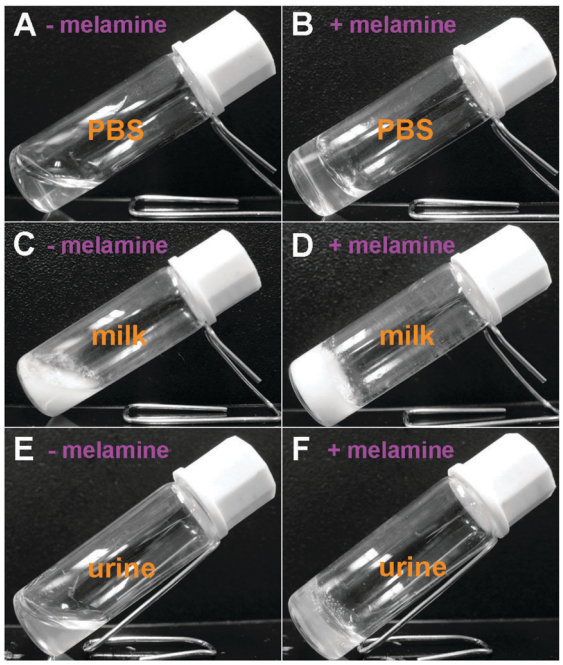

Fig. 1 Optical images of the solutions of Nap-FFYGK-CA without (left images) or with (right images) 35 ppm, 20 ppm, 20 ppm melamine in PBS (A and B), milk (C and D), and urine (E and F), respectively.

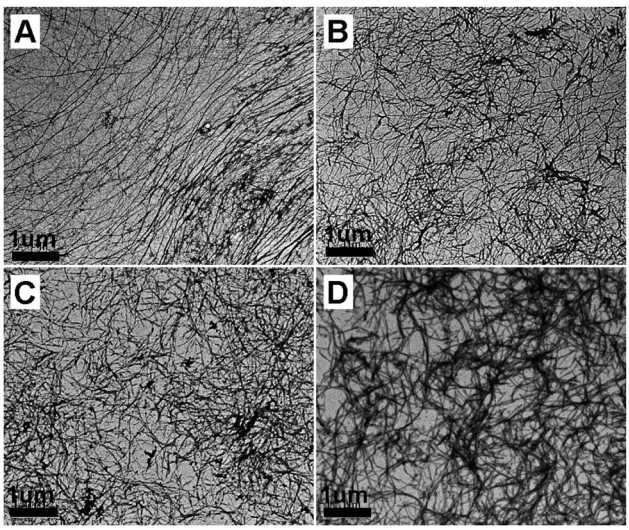

Fig. 2 TEM images of (A) solution of Nap-FFYGK and gels with different equiv. of melamine: (B) 2 equiv., (C) 4 equiv. and (D) 16 equiv.

of melamine in these two samples. There are proteins and urea in milk and urine, respectively, which may interfere with the hydrogelation. As shown in Fig. 1C, milk without melamine cannot form a hydrogel at the concentration of 0.5 wt%. With the addition of melamine above the concentration of 20 ppm, a hydrogel would form within 30 min (Fig. 1D). Similar observations were achieved for melamine tainted urine, and the minimum detection concentration of melamine in urine was also about 20 ppm (Fig. 1E and F). Compared with other melamine detecting systems, which need to extract melamine from the samples, our

method was much simpler that the analytes could be detected directly by the naked eye. Such a convenient method is very promising for the detection of melamine where no instrument is accessible.

We then used transmission electron microscopy (TEM) to characterize the morphology of self-assembled structures in the solution and hydrogels with different amounts of melamine. As shown in Fig. 2A, the compound Nap-FFYGK-CA self-assembled into filamentous structures with the diameter of about 25 nm. The density of the cross-linking point was low probably due to the lack of strong interactions between fibers. In the presence of melamine, we observed higher densities of the cross-linking point in the gel samples (Fig. 2B–D and Fig. S5–S8, ESI†). Compared with the fibers in the solution without melamine, the diameter of those in gels was bigger. Many reports have demonstrated that the hydrogen bonding and π–π stacking between melamine could assist the formation and tune the morphology of self-assembled nanostructures.[19] In our study, we also observed that the diameter of fibers became bigger in the presence of more melamine and it was about 33, 40, 48, 53, 58, 68, and 77 nm in the presence of 0.25, 0.5, 1, 2, 4, 8, 16 equivalent of melamine, respectively (Fig. S9, ESI†). It is well-known that in order to form supramolecular hydrogels, there should be strong or at least medium interactions between self-assembled nanostructures.[20] Our previous study had also showed that using a recombinant protein with multiple binding sites, we could enhance the inter-fiber interaction by the specific protein–peptide interaction for hydrogelations.[21] In this study, with the assistance of CA–melamine complexation, we could enhance the interaction between supramolecular nanofibers, resulting in the formation of stable 3D networks and hydrogels.

We further investigated the mechanical property of hydrogels by rheology. The hot aqueous solutions containing Nap-FFYGK-CA and different amounts of melamine were directly transferred to the rheometer. After 2 hour incubation to achieve stable hydrogels, we performed a dynamic strain/frequency sweep. The value of the storage moduli (G', elasticity) of all samples was bigger than that of their corresponding loss moduli (G'', viscosity), suggesting that all samples behaved as viscoelastic materials. The hydrogels showed bigger G' values with the increased amounts of melamine when the equivalent of melamine was lower than 2 (Fig. 3 and Fig. S10–S12, ESI†). For instance, the G' value reached 8728 Pa at the frequency value of 0.1 rad s^{-1} for the gel with 2 equivalent of melamine, which was about 35 times bigger than that with 0.25 equivalent of melamine. However, when the amount of melamine was more than 2 equivalent, the G' value of resulting hydrogels dropped (Fig. 3). These results indicated that the mechanical property of the hydrogels might be regulated by changing the molar ratios of melamine. As the mechanical properties of hydrogels are critical for their biological applications, a hydrogel that can finely tune the mechanical properties is highly desired.[22]

Based on the above observations, we proposed possible interactions between melamine and supramolecular nanofibers in the hydrogels. As shown in Fig. 4, Nap-FFYGK-CA itself could self-assemble into aligned nanofibers with a CA moiety at their surfaces. With low density of cross-linking points, these nanofibers can only form solutions. Assisted by the hydrogen bonds between melamine and the CA derivative, the nanofibers entangled with each other and formed stable 3D networks for hydrogelations. When the concentration of melamine was low, it served as the cross-linker. With the increase of melamine, more cross-linking points formed, resulting in higher G' values of the gels. However, melamine could also stack with each other. When an excess amount of melamine was present in the gel, melamine assembly formed at the surface of nanofibers, leading to the increase of diameter of nanofibers. In such situation, melamine assembly served as the cross-linkers to form fiber networks. Since the interaction between melamine was not so strong as that between melamine and CA, the mechanical property of gels with an excess amount of melamine decreased.

In summary, we reported a supramolecular hydrogel-based detection system for melamine. Using this method, melamine could be directly visualized by the naked eye in milk and urine without pre-extraction processes and the need of any instruments. Although the minimum detection concentration of melamine for our system is a little bit higher than the safety limits (2.5 ppm in the USA and EU and 1 ppm in China), we have provided a simple, real-time, and on-site way to detect melamine at the concentrations of higher than 20 ppm in foods and in biological samples.

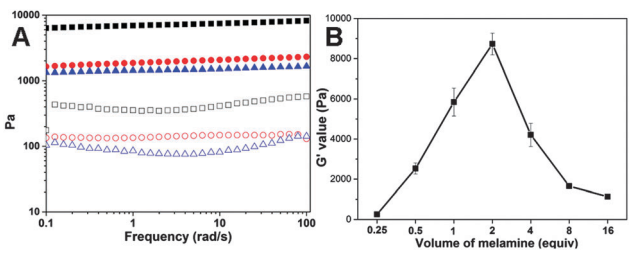

Fig. 3 (A) Dynamic frequency sweep at the strain of 0.1% of the hydrogels with addition of 0.5 (circles), 2 (squares) and 8 (triangles) equiv. of melamine (filled symbols: G' and open symbols: G''), and (B) the G' value in the mode of dynamic frequency sweep at the strain of 0.1% of the gels with different equivalents of melamine.

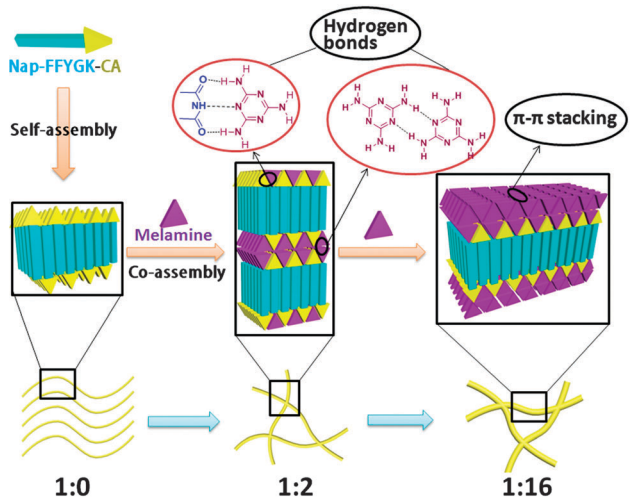

Fig. 4 Possible molecular arrangements in self-assembled nanofibers of Nap-FFYGK-CA and in gels with different amounts of melamine.

Our method is useful for the detection of high concentration of melamine in remote places without instruments.

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