Using Congo red to report intracellular hydrogelation resulted from self-assembly of small molecules†

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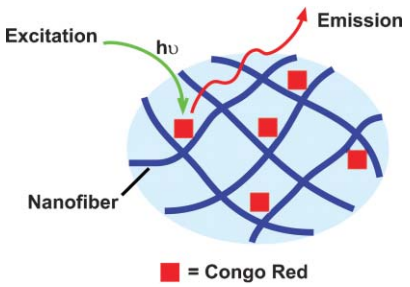
This communication reports the use of Congo red to stain the nanofibers of self-assembled small molecules for assaying intracellular supramolecular hydrogels, which provides a convenient method to explore molecular self-assembly inside cells.

Supramolecular hydrogels,[1,2] formed by hydrogelators self-assembling into three-dimensional networks of nanofibers to trap water, have shown promise for biological applications such as drug delivery,[3] biosensing,[4] tissue engineering,[5] and wound healing.[6] Though extracellular formation and applications of supramolecular hydrogels have achieved considerable development, intracellular hydrogelation to create artificial nanostructures and their potential applications are just beginning to be explored as a nascent direction in supramolecular hydrogel research. Recently, we have demonstrated that small molecular hydrogelators, after being produced inside cells, could form nanofibers intracellularly and result in hydrogelation to induce cell death or to inhibit cell growth.[7] To confirm these nanofibers of hydrogels had formed intracellularly, we used transmission electron microscopy (TEM), which required laborious sample preparation and sophisticated instrumentation. Therefore, a quick, convenient assay would greatly help the research on intracellular hydrogelation by eliminating the need for expensive instruments. Here we report the use of Congo red (**1**) to stain the nanofibers of intracellular supramolecular hydrogels inside two types of mammalian cell and one strain of bacterium. After being stained by Congo red, the intracellular hydrogel resulting from the self-assembly of Nap–Phe–Phe motif retains the Congo red to exhibit red fluorescence as the specific signal (Scheme 1). This Congo red assay, as an effective and easy method for determining intracellular hydrogelation, should also assist the exploration of the intracellular self-assembly of small molecules.

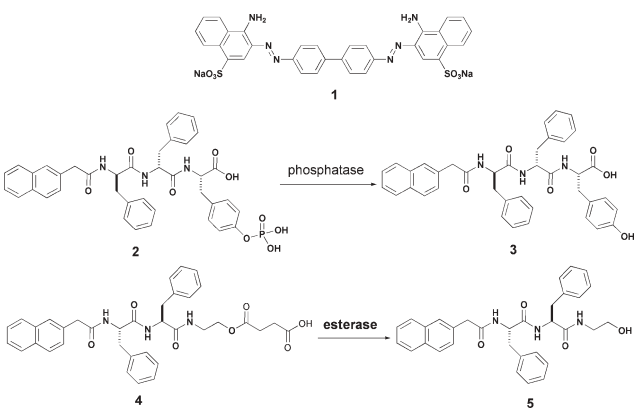
Several diseases, including Alzheimer's disease, spongiform encephalopathies, familial amyloid polyneuropathy, and type II diabetes, are associated with proteins that undergo abnormal conformational changes and polymerize into amyloid fibers.[8] A distinctive feature of amyloid fibers is the predominant presence of



Scheme 1 Illustration of Congo red to stain nanofibers.

β-sheets[9] that can bind to Congo red.[10] Among many kinds of supramolecular hydrogels developed, a considerable proportion of them are peptide derivatives.[11,12] Similar to amyloid fibers, these peptide-based supramolecular hydrogelators also self-assemble into β-sheet-like nanostructures.[13] Because Congo red has good cell permeability and inhibits the formation of β-amyloid fibrils inside cells,[14] Congo red might be able to selectively stain this type of nanofiber formed in supramolecular hydrogelation. Based on this assumption, we chose to use Congo red to stain these hydrogels for identifying intracellular hydrogelation. Scheme 2 gives the molecular structure of the sulfonated azo dye, Congo red (**1**). Like its binding to amyloid fibers, the sulfonate groups of **1** interact with the hydrophilic part of the assembly of hydrogelators, while the hydrophobic core of **1** binds to the hydrophobic part of the nanofibers in the hydrogel. Being excited by a green light (510–560 nm), **1** gives a red fluorescent emission peak at 616 nm that is suitable for imaging the process inside cells (Fig. S1†).

We used Nap–D-Phe–D-Phe–tyrosine phosphate (**2**) and Nap–Phe–Phe–$NH(CH_2)_2OCO(CH_2)_2COOH$ (**4**) as the precursors for



Scheme 2 Molecular structures of Congo red (**1**), the precursors (**2**, and **4**), and the hydrogelators (**3**, and **5**).

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enzymatic hydrogelation to test the binding of **1** to the self-assembled nanofibers for the following reasons. (i) Enzymatic hydrogelation is an effective route to create self-assembled nanostructures of small molecules.[11] (ii) Nap–Phe–Phe and amyloid fibers share a common motif, Phe–Phe, that tends to self-assemble into β-sheet or β-sheet-like structures that bind to Congo red. (iii) Compounds **2** and **4** are the substrates of two endogenous enzymes, phosphatase and esterase, respectively. The transformations of **2** and **4** (the precursors) to **3** and **5** (the hydrogelators) are representative processes of enzymatic intracellular hydrogelation of small molecules.

We first tested the binding of Congo red to the self-assembled nanofiber of the hydrogel extracellularly. After the addition of alkaline phosphatase to the PBS buffer solution containing **2** (0.6 wt%), a transparent hydrogel forms within minutes (Fig. 1A, inset). A TEM image of the cryo-dried Gel **I** shows that the nanofibers in Gel **I** have a length of over 10 μm, a width of about 10–20 nm, and an average pore size of the network of about 100 nm. Similarly, the addition of esterase to the solution containing **4** (0.5 wt%) at 37 °C results in Gel **II**, whose nanofibers have a length of over 10 μm, a width of about 10–20 nm, and an average pore size of the network of about 200 nm (Fig. S2†). The diameters of the nanofibers in Gel **I** and Gel **II** are within the same range as those of β-amyloid fibers formed *in vitro* and *in vivo* (5–17 nm) that can be stained by Congo red.[10] After the Congo red stock was added to Gel **I** at a calculated final concentration of 10 μM, the transparent colorless Gel **I** was slowly stained by Congo red solution and turned a bright red color (Fig. 1B, inset). TEM revealed that there were black particles adhering to the nanofibers of Gel **I** after Congo red staining (Fig. 1B). A high resolution TEM image of Congo red stained Gel **I** and its EDX analysis revealed that the dark particles attached to the nanofibers and dispersed in the hydrogel matrix were Congo red (because they contain sulfur element) (Fig. S3†). In addition, circular dichroism (CD) spectra of the hydrogels showed a positive peak at 195 nm and a negative peak center at 225 nm (exciton splitting of the peptide π–π* transition) for Gel **I** and a positive peak at 198 nm and a negative peak at 214 nm for Gel **II**, indicating that most of **3** and **5** took a β-sheet-like arrangement in the gel phase (Fig. S1†).

To prove the specificity of the Congo red staining, we used a polymeric hydrogel made of cross-linked polyacrylamide as the control. Without Congo red staining, both the supramolecular hydrogels and the polyacrylamide hydrogel were colorless. After Congo red staining, a transparent hydrogel with bright red color was obtained (Fig. S4B†). The Congo red remained in the supramolecular hydrogel after being washed with PBS buffer three times. On the other hand, the polyacrylamide hydrogel returned to colorless after the same washing process (Fig. S4C†). A confocal laser scanning micrograph showed the fiber-like features in the Congo red stained Gel **I** (Fig. 2B), confirming that Congo red was able to bind the nanofiber in the hydrogel.

After validating Congo red as the dye for Nap–Phe–Phe based supramolecular hydrogel specifically, we tested the staining of self-assembled nanofibers of hydrogels inside cells. We used HeLa cells to investigate the intracellular gelation of **2** because HeLa cells could over-express phosphatase.[15] Presumably, the over-expressed phosphatase can dephosphorylate the up-taken hydrogel precursor (**2**) to accumulate enough hydrophobic hydrogelator (**3**), thus leading to self-assembled nanofibers and forming the hydrogel intracellularly. As shown in Fig. 3, Congo red cannot stain the cells when HeLa cells were treated with **2** for 24 h and at a concentration (250 μM) below the IC_{50} (410 μM) (Fig. 3E), likely due to there being too few hydrogelators to induce intracellular hydrogelation to result in cell death or inhibit cell growth. When the concentration of **2** (1 mM) exceeded the IC_{50}, the Congo red-stained HeLa cells showed strong red fluorescence (Fig. 3F). Apparently, most of the red fluorescence localized at the region of the nucleus (Fig. S6†), suggesting that the nanofibers might form around the nucleus. Similar to the staining of HeLa cells treated with **2**, Congo red cannot stain the HepG2 cells treated with **4** for 24 h and at a concentration (160 μM) below the IC_{50} (341 μM) (Fig. 3K). When the concentration of **4** (640 μM) exceeded the IC_{50}, the Congo red-stained HepG2 cells also showed strong red fluorescence (Fig. 3L).

We also used **1** to stain the cells that were treated with 1% of Triton X-100 as the necrosis model. Both the necrotic models of HeLa cells and HepG2 cells showed no red fluorescence after the staining of Congo red (Fig. 3D and 3J). This result excludes the possibility that necrosis of cells causes the staining of Congo red and confirms that the strong red fluorescence in Fig. 3F and 3L is the result of the staining of nanofibers in the supramolecular hydrogels by Congo red.

After confirming that Congo red selectively assays intracellular hydrogelation in mammalian cell lines, we also used Congo red to stain phosphatase overexpressed *E. coli* after they were incubated with **2** for 24 h. Congo red could only stain phosphatase overexpressed *E. coli* that were incubated with **2** (Fig. 3R), agreeing with the formation of the hydrogel of **3**. Because of the short life cycle, some of the *E. coli* had not built up enough concentration of **3** to form an intracellular hydrogel, and they show little fluorescence as the result of the staining of Congo red. Nevertheless, the observed fluorescence clearly suggests that

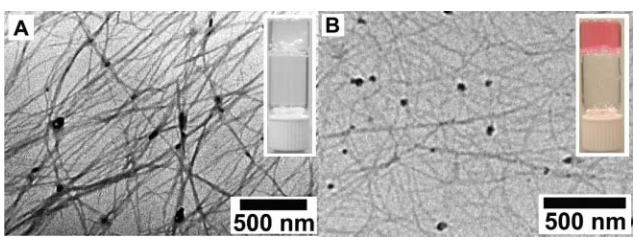

Fig. 1 TEM images of (A) Gel **I** (inset: optical image) formed by **3** *via* enzymatic gelation of **2** in PBS buffer (conc. = 0.6 wt%, pH = 7.4); and (B) Congo red stained Gel **I** (inset: optical image).

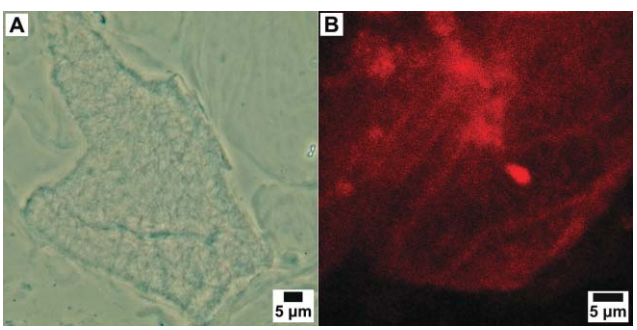

Fig. 2 (A) Phase contrast micrograph of Congo red stained Gel **I**; and (B) confocal laser scanning micrograph of Congo red stained Gel **I**.

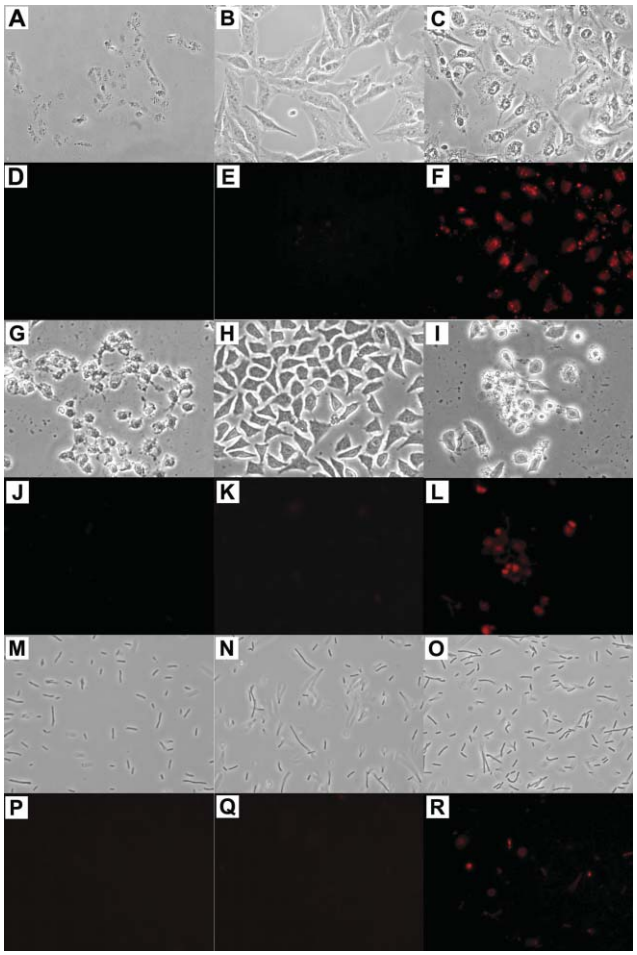

Fig. 3 (A, B, C) Optical images (objective: 20 ×) of HeLa cells stained by Congo red: (A) Triton X-100 treated for 1 min before staining; (B) 250 μM and (C) 1 mM of **2** treated for 24 h; (D, E, F) corresponding fluorescence images of (A, B, C). (G, H, I) Optical images (objective: 20 ×) of HepG2 cells stained by Congo red: (G) Triton X-100 treated for 1 min before staining; (H) 160 μM and (I) 640 μM of **4** treated for 24 h; (D, E, F) corresponding fluorescence images of (G, H, I). (M, N, O) Optical images (objective: 100 ×) of phosphatase overexpressed *Escherichia coli* stained by Congo red: (M) Control; (N) 1.73 μM and (O) 3.46 μM of **2** treated for 24 h at 18 °C; (P, Q, R) corresponding fluorescence images of (M, N, O).

Congo red can identify the formation of nanofibers of hydrogels within the bacteria.

In summary, we have successfully demonstrated that Congo red serves as an assay to stain the peptide-based supramolecular hydrogel extra- and intracellularly. Although only two types of hydrogelator, two cancer cell lines and one strain of bacterium were investigated in this work, it is conceivable that Congo red can stain other supramolecular hydrogels intracellularly because Hamachi *et al.* have shown the amphiphilic nanofibers in supramolecular hydrogels can dramatically enhance the fluorescence.[2] To improve the specificity of this type of assay, other chromophores or fluorophores that show definitive features of aggregation[16] or molecular imaging agents[17] are worthy to be explored.

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