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A supramolecular hydrogelator of curcumin†

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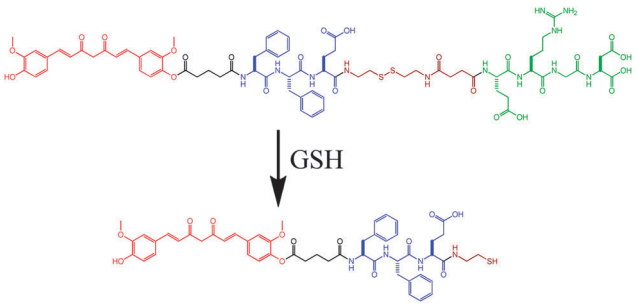
Here we report on the first supramolecular hydrogelator of curcumin and the evaluation of its inhibition capacity towards cancer cells and tumor growth.

Supramolecular hydrogels[1] of therapeutic agents have recently attracted extensive research interest as drug delivery carriers because of their several advantages,[2] such as the high and controllable drug loading, sustained and responsive drug release properties, and good biocompatibility.[3] Supramolecular hydrogels are formed by hydrogelators *via* non-covalent interactions.[4] Up to now, several kinds of therapeutic agents including anti-cancer,[5–7] anti-bacteria,[8] and anti-inflammatory drugs[6,9] have been converted into hydrogelators through conjugation with small molecules, especially peptides.[10] The resulting hydrogels can be used directly as injectable hydrogels for the topical treatment[11] or after dilution as nanofiber dispersions[12] by intravenous injection, and they have shown constant release of therapeutic agents and excellent inhibition capacity towards cancer cells[13,14] and bacteria.[15] For their application in cancer therapy, anti-cancer drugs taxol[7,12,14,16] and camptothecin[5,6] have been converted into hydrogelators by several groups. In order to expand the scope of hydrogelators of anti-cancer drugs and develop more of these hydrogels for combinatory therapy, there is a need to develop hydrogelators of other anti-cancer drugs. In this study, we report on the first supramolecular hydrogelator of the anti-cancer drug curcumin (Cur).

Cur has been widely used as a food additive (*e.g.* curry) and has shown anti-bacterial[17] and anti-cancer[18] properties. In order to improve its solubility in aqueous solutions and its bioavailability, it has been formulated into micelles and nanospheres, or incorporated into liposomes or polymeric hydrogels.[19] However, there is no supramolecular hydrogelator of Cur. We planned to develop a hydrogelator of it and generate a hydrogel for its delivery. We therefore designed the molecule Cur-FFE-ss-ERGD, shown in Scheme 1, as a pro-gelator because the dipeptide diphenylalanine (FF) has been widely used to construct supramolecular hydrogelators. We also demonstrated that disulfide bond reduction was a biocompatible method for hydrogelation.[7] Therefore we believed that, upon the addition of glutathione (GSH), the pro-gelator would be converted to a possible hydrogelator, thus resulting in hydrogelation.



Scheme 1 The chemical structures of pro-gelator Cur-FFE-ss-ERGD and possible gelator catalyzed by glutathione (GSH).

Following our previously published procedure,[7] we first prepared Fmoc-CS which contains a disulfide bond. The compound was then directly used for standard Fmoc solid phase peptide synthesis to produce the peptide FFE-ss-ERGD. We then prepared the Cur derivative with a carboxylic acid by reacting Cur with glutaric anhydride, which coupled with the peptide to achieve the title compound. The pure compound was obtained by reverse phase high performance liquid chromatography (HPLC) and it showed a very good water solubility of up to 5 wt% (50 mg mL^{-1}) in phosphate buffer solution (PBS, pH = 7.4).

We then tested its gelation ability by disulfide bond reduction. As shown in Fig. 1A, the addition of 4 equiv. of

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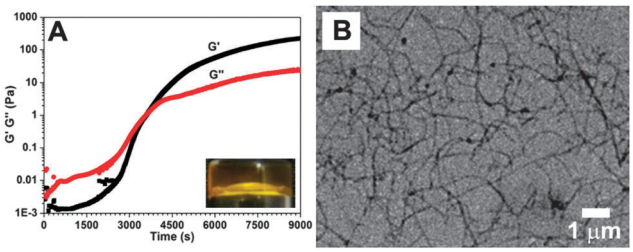

Fig. 1 (A) Rheology with the dynamic time sweep mode for a PBS solution containing 0.5 wt% of the pro-gelator with 4 equiv. GSH (inset: optical image of the formed gel) and (B) a TEM image of the formed gel.

GSH to a PBS solution of the pro-gelator (0.5 wt%, 5 mg mL^{-1}) resulted in the formation of a clear yellowish hydrogel (Fig. 1A, inset) after 1.5 h at 37 °C. We then characterized the mechanical properties of the hydrogel by rheology. As shown in Fig. 1A, when GSH was added to the pro-gelator solution, the value of the storage modulus (elasticity or G') became more dominant than the loss modulus (viscosity or G'') after about 1 h. Both G' and G'' exhibited a weak frequency dependency from 0.1 to 100 rad s^{-1} (Fig. S4, ESI†), suggesting an elastic network in the gel. We then characterized the nanostructures in the hydrogel by transmission electron microscopy (TEM, Fig. 1B). We observed filamentous structures in the gel and the diameter of the fibrils was about 25–35 nm. These uniform and flexible fibrils were longer than 2 μm and entangled with each other to form a network for the gel formation.

We therefore monitored the release profile of Cur from the gel *in vitro* under physiological temperature conditions (at 37 °C). We added 0.25 mL PBS buffer solution on top of 0.25 mL of the gel formed from 0.5 wt% Cur-FFE-ss-ERGD at a 24 h time point. The upper solution was completely removed at desired time intervals to measure the accumulated amount of Cur released from the gel and a fresh PBS buffer solution (0.25 mL) was then added. We only observed the release of the original Cur and no Cur derivatives were released from the gel, suggesting hydrolysis of the ester bond. As shown in Fig. 2A, the gel exhibited a constant release profile at a rate of about 0.8 μg mL^{-1} per hour during the 24 h experimental period. About 1.9 μg mL^{-1} Cur was released during the 24 h. These observations suggest its big potential for the sustained and long term delivery of Cur.

We also obtained the IC_{50} value of the pro-gelator, gelator, peptide, and Cur against HepG2, HeLa, and MCF-7 cells. After incubating the cells with different compounds at different concentrations for 48 h, an MTT assay was performed. As shown in Fig. 2B, the pro-gelator exhibited an IC_{50} value of 8.1, 8.4, and 9.5 μM against HepG2, MCF-7 and HeLa cells, respectively, which is very similar that of Cur. The peptide without Cur showed no obvious toxicity to the cells at a concentration of 5 mM. The gel showed a decreased inhibition capacity to three cells and its IC_{50} value was 27.8, 52.9, and 53.2 μM against HepG2, HeLa, and MCF-7 cells, respectively. These observations suggest a superior inhibition capacity of the pro-gelator compared to the gelator in the nanofiber form.

In order to understand the superior inhibition capacity of the pro-gelator than the gelator, we obtained confocal fluorescence microscopy images of MCF-7 cells treated with a solution of the pro-gelator and the gelator in the form of nanofibers. Fig. 3 shows overlay images of the MCF-7 cells at a 4 h time point (excitation wavelength = 488 nm). The pro-gelator was distributed evenly in the cytoplasm of the cells, as indicated by the bright green fluorescence in the entire cytoplasm of the cells (Fig. 3A and Fig. S7, ESI†). However, we observed much weaker fluorescence in cells treated with the nanofibers and the fluorescence signal was not evenly distributed in the cells (Fig. 3B and Fig. S7, ESI†). We then used liquid chromatography-mass spectrometry (LC-MS) to determine the concentration of the compounds in the cells when pro-gelator and gelator solutions were used to treat the cells. We found that the concentration of the gelator was 6.8, 5.3, and 4.9 times higher than that of the pro-gelator in MCF-7, HeLa, and HepG2 cells, respectively (Fig. S8, ESI†), indicating a much higher cellular uptake of the nanofibers than of the pro-gelator. It has been demonstrated that self-assembled molecules have higher cellular uptake than single molecules.[20] The weaker fluorescence in cells treated with the nanofibers was due to the well-known phenomenon of aggregation induced quenching. These observations also suggest that the nanofibers changed the distribution of Cur, thus resulting in its less reduced inhibition capacity to cancer cells.

We opted to compare the *in vivo* anti-cancer capacity of the pro-gelator solution and the solution of nanofibers in a mouse tumor model (4T1-luciferase breast tumors in the mammary fat

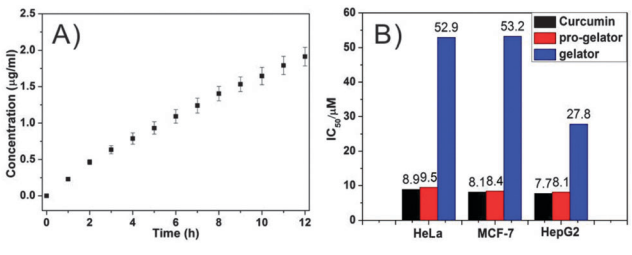

Fig. 2 (A) The accumulative amount of curcumin released from the gel formed from a PBS buffer containing 0.5% wt pro-gelator and (B) cytotoxicity of the pro-gelator, the gel, and curcumin against HepG2, HeLa, and MCF-7 cells.

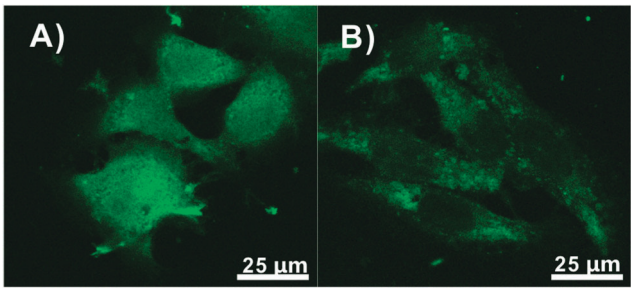

Fig. 3 Confocal fluorescence microscopy images of MCF-7 cells treated with (A) the pro-gelator and (B) the formed nanofibers containing 25 μM curcumin (excitation wavelength = 488 nm) at a 4 h time point.

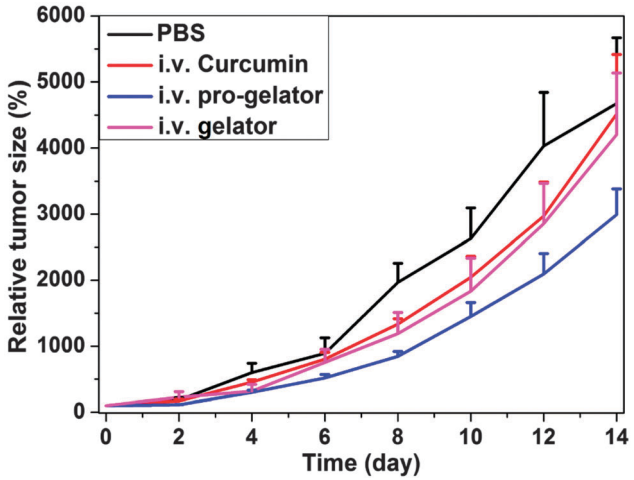

Fig. 4 Pro-gelator and nanofiber solutions inhibit xenografted mouse breast tumor (4T1-luciferase) growth *in vivo* (the gel was first diluted in PBS solution and then administrated into the caudal vein after the tumor size had reached about 13 mm^3, $n = 6$).

pad of female mice). When the volume of breast tumors reached about 13 mm^3, we injected the same dosage (2.5 mg kg^{-1} of Cur × 4 every other day, Cur was dissolved in an excipient mixture of polyethylene glycol 400, propylene glycol, and polysorbate 80 (40 : 58 : 2)) of different formulations of Cur into mice through the caudal vein. As shown in Fig. 4, the solution of nanofibers exhibited a similar anti-tumor growth efficacy to Cur. The pro-gelator showed an enhanced anti-tumor growth capacity over the nanofibers and Cur. The final volume of the tumors was about 4675, 4518 ($^*P = 0.0446$), 4207 ($^*P = 0.0233$), and 2992% ($^{***}P < 0.0001$) higher than the original volume of the tumors (13 mm^3) for the PBS control group, Cur, the nanofibers, and the pro-gelator, respectively. There was no obvious body weight loss in the groups of mice administrated with different forms of Cur (Fig. S9, ESI†), compared to the control group of mice treated with PBS. These results, in combination with the results giving the *in vitro* inhibition capacity to cells, suggest that the pro-gelator is a more promising candidate than the gelator in nanofibers for cancer therapy.

In summary, a new hydrogelator based on Cur was reported in this study. The resulting hydrogel formed by disulfide bond reduction and could sustainably release the original Cur through ester bond hydrolysis. Though the cellular uptake of the nanofibers of the Cur–peptide conjugate was much higher than that of solutions of the pro-gelator, the nanofibers possessed a lower potency to inhibit cancer cells *in vitro* and *in vivo* than the pro-gelator solution. Therefore, the hydrogel might only be applied for the topical treatment of cancers. The results also indicate that, in order to achieve better inhibition capacity of Cur nanofibers on cancer cells, the nanofibers are required to be responsive to a pH change after endocytosis and must dissociate into single molecules. Our study provides useful information to design nano-materials to deliver the anti-cancer drug, curcumin.

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