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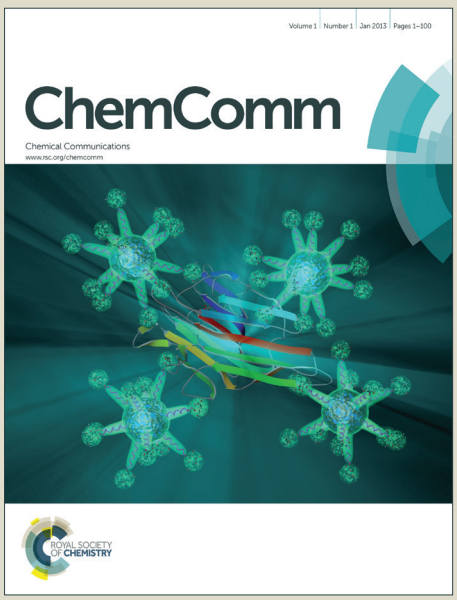

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ARTICLE TYPE

The first supramolecular hydrogelator of curcumin

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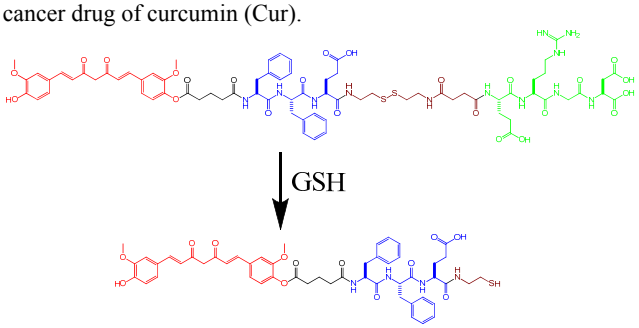
Here we reported on the first supramolecular hydrogelator of curcumin and the evaluation of its inhibition capacity to cancer cells and tumor growth.

Supramolecular hydrogels[1] of therapeutic agents have attracted extensive recent research interests because of their several advantages as drug delivery carriers,[2] such as the high and designable drug loadings, sustained and responsive drug release property, 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, 6, 7] anti-bacteria,[8] and anti-inflammatory drugs[6, 9] have been developed 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] for intraveneous injection, and they have showed constant release properties of therapeutic agents and excellent inhibition capacities to cancer cells[13, 14] and bacteria.[15] For their application in cancer therapy, anti-cancer drugs of taxol[7, 12, 14, 16] and camptothecin[5, 6] have been developed into hydrogelators by several groups. In order to expand the scope of hydrogelators of anti-cancer drugs and develop more such 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 of curcumin (Cur).



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

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

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

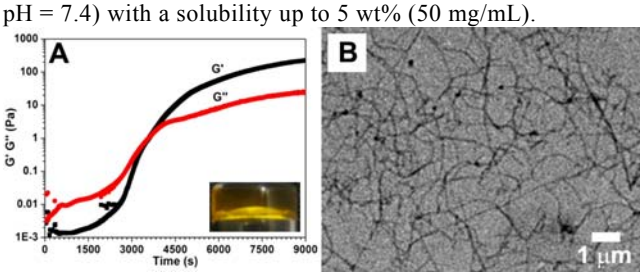


Fig. 1. A) Rheology with the mode of dynamic time sweep for a PBS solution containing 0.5 wt% of the pro-gelator with 4 equiv. of GSH (insert: optical image of the formed gel) and B) a TEM image of the formed gel

We then tested its gelation ability by disulfide bond reduction. As shown in Fig. 1A, the addition of 4 equiv. of GSH to a PBS solution of the pro-gelator (0.5 wt%, 5 mg/mL) resulted in the formation of a clear yellowish hydrogel (Fig. 1A, insert) after 1.5 h at 37 °C. We then characterized the mechanical properties of the hydrogel by rheology. As shown in Fig. 1A, when adding GSH into the solution of pro-gelator, the value of the storage modulus (elasticity or G') became dominating the loss modulus (viscosity or G") after about 1 h. Both the G' and G'' exhibited weak frequency dependences

from 0.1 to 100 rad s^{-1} (Fig. S-4), suggesting an elastic network in the gel. We then characterized the nanostructures in the hydrogels by transmission electron microscopy (TEM, Fig. 1B). We observed filamentous structures in the gel and the diameter of fibrils was about 25–35 nm. These uniform and flexible fibrils were longer than 2 μm and they entangled with each other to form a network for the gel formation.

We therefore monitored the releasing profile of Cur from the gel *in vitro* at physiological temperature condition (at 37℃). We added a 0.25 mL of PBS buffer solution on the top of 0.25 mL of gel formed from 0.5 wt% of Cur-FFE-ss-ERGD at 24 h time point. The upper solutions were totally taken out at desired time intervals for the measurement of accumulating released amount of Cur from the gel and a fresh PBS buffer solution (0.25 mL) was then added. We only observed original Cur and no Cur derivatives being released from the gel, suggesting the 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 per hour during the 24 h experimental period. There was about 1.9 μg/mL of Cur being released during the 24 h. These observations suggested its big potential for sustained and long term delivery of Cur.

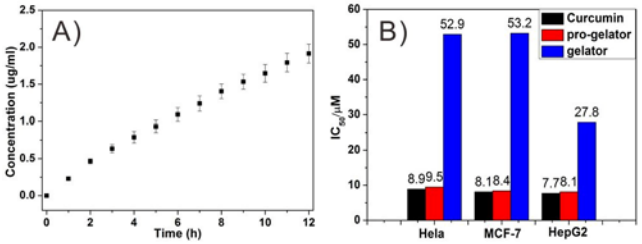


Fig. 2. A) Accumulative release amount of curcumin from the gel formed from PBS buffer containing 0.5% wt pro-gelator and B) Cytotoxicity of pro-gelator, the gel, and curcumin against HepG2, HeLa, and MCF-7 cells.

We also obtained the IC_{50} value of the pro-gelator, gelator, peptide, and Cur against HepG2, HeLa, and MCF-7 cells. After incubating cells with different compounds at different concentrations for 48h, the 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, HeLa, and MCF-7 respectively, which was very similar that of Cur. The peptide without Cur showed no obvious toxicities 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 to HepG2, HeLa, and MCF-7, respectively. These observations suggested a better inhibition capacity of the pro-gelator than the gelator in nanofibers.

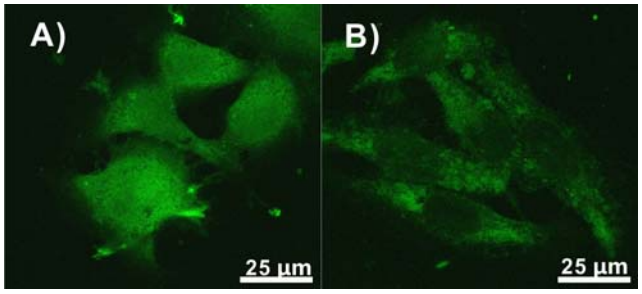


Fig. 3. Confocal fluorescence microscopy images of MCF-7 cells treated with A) the pro-gelator and B) the formed nanofiber at 4 h time point containing 25 μM curcumin (excitation wavelength = 488 nm).

In order to understand the better inhibition capacity of the pro-gelator than the gelator, we obtained the confocal fluorescence microscopy of MCF-7 cells treated with the solution of the pro-gelator and the gelator in the form of nanofibers. Fig. 3 showed the overlay images of MCF-7 cells at 4 h time point (excitation wavelength = 488 nm). The pro-gelator distributed evenly in the cytoplasma of cells, as indicating by the bright green fluorescence in the whole cytoplasma of cells (Fig. 3A and Fig. S-7). 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. S-7). We then used the LC-MS to determine the concentration of compounds in cells when pro-gelator and gelator solutions were used to treat the cell, respectively. 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. S-8), indicating much higher cellular uptake of the nanofiber than that of the pro-gelator. Self-assembled molecules had been demonstrated to have higher cellular uptake than single molecule.[20] The weaker fluorescence in cells treated with the nanofibers was due to the well-known phenomenon of aggregation caused quenching. These observations also suggested that the nanofibers changed the distribution of Cur, thus resulting in its less inhibition capacity to cancer cells.

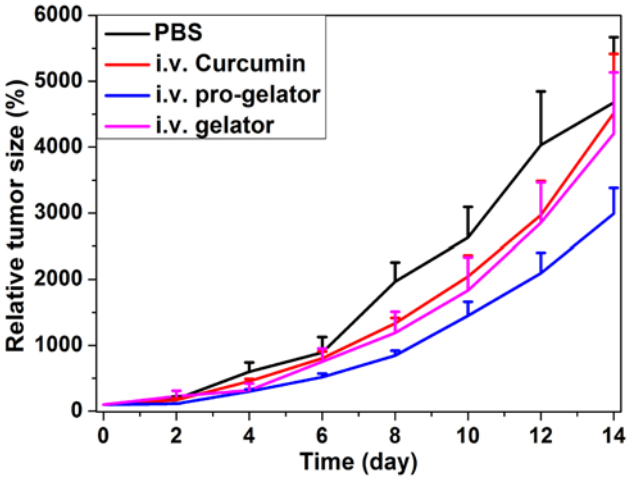


Fig. 4. Pro-gelator and nanofiber solutions inhibit xenografted mouse breast tumor (4T1-luciferase) growth *in vivo* (the gel was firstly diluted in PBS solution and then administrated into the caudal vein after the tumor size reaching about 13 mm^3, n = 6).

We opted to compare the *in vivo* anti-cancer capacity of the pro-gelator solution and the solution of nanofibers in the mice tumor model (4T1-luciferase breast tumors in mammary fat pad of female mice). When the volume of breast tumors reached about 13 mm^3 , we injected the same dosages (2.5 mg/kg 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 the Cur. The final volume of tumors was about 4675, 4518 (*P=0.0446), 4207 (*P=0.0233), and 2992% (***P<0.0001) bigger than the original volume of tumors (13 mm^3) for the PBS control group, Cur, nanofibers, and pro-gelator,

respectively. There were no obvious body weight losses in groups of mice administrated with different forms of Cur (Fig. S-9), compared to the control group of mice treated with PBS. These results, in combination with their *in vitro* inhibition capacities to cells, suggested that the pro-gelator was 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 hydrogels formed by disulfide bond reduction could sustainedly release original Cur through the ester bond hydrolysis. Though the cellular uptake of nanofibers of Cur-peptide conjugate was much higher than that of solutions of the pro-gelator, the nanofibers possessed a less 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 capacities of Cur nanofibers on cancer cells, the nanofibers are required to be responsive to pH change after endocytosis and must dissociate into single molecule. Our study provides useful information to design nano-materials to deliver anti-cancer drug, curcumin.

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Notes and references

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