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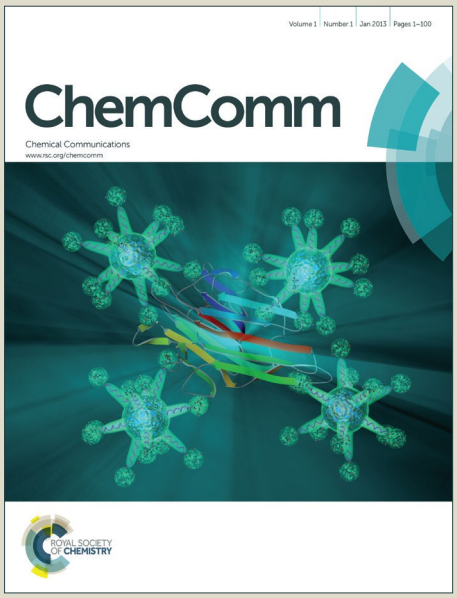

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COMMUNICATION

Galactose-decorated light-responsive hydrogelator precursor for selectively killing cancer cells

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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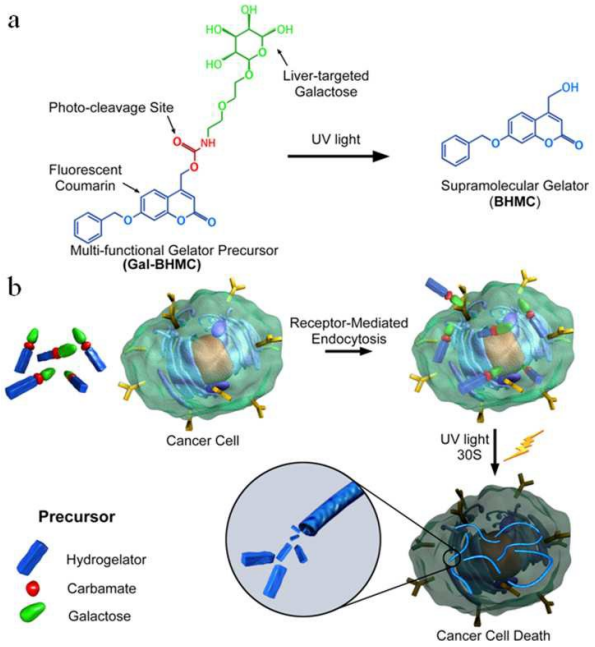
A multi-functional gelator precursor with high photosensitivity is rationally designed, which can not only selectively target hepatocellular carcinoma (HCC) cells, but also form intracellular self-assembly triggered by light and subsequently inducing cell death. The study develops a methodology to design biologic targets of supramolecular self-assembly under external stimuli for cancer research.

Intracellular self-assembly of small molecules is a hot topic for its promising biomedical applications in tumor diagnostics[1-2] and therapeutics.[3] Recently, enzyme-responsive peptide-based precursors have been frequently reported since they could penetrate cell membranes and self-assemble into nanofibers inside living cells,[4-8] which could affect cellular functions and further induce cell apoptosis due to the promiscuous interactions between nanofibers and cytoskeletal proteins.[9] However, the release of gelators from their precursors by enzyme (an internal stimulus) is still difficult to be switched on and off at will.[10] Furthermore, these gelator precursors usually passively attack cancer cells and could induce inefficient tumor cell uptake. Whereas, this may be circumvented by grafting specifically recognized ligands onto precursors, which can allow the active target to cancer cells through the specific interaction between ligands and receptors (over-expressed by cancer cells).[11] Thus, it is very necessary to develop an innovative designing methodology to explore functional gelator precursors that can not oly selectively target cancer cells, but also precisely release gelators under external stimuli, which is still a question remained to be answered so far.



Scheme 1. a) Molecular structure of precursor Gal-BHMC and its conversion to gelator BHMC upon UV light irradiation. b) Schematic demonstration of specific targeting of Gal-BHMC onto cancer cells and cell death induced by intracellular self-assembly triggered by light.

Herein, a multi-functional gelator precursor (Gal-BHMC) with high photosensitivity is rationally designed (Scheme 1a), which can not only selectively target hepatocellular carcinoma (HCC) cells, but also release gelators under light irradiation, leading to the intracellular supramolecular self-assembly and subsequently inducing cell death. Gal-BHMC contains three different functional groups: a galactose unit, a photo-responsive carbamate bond of (coumarin-4-yl)-methyl derivative,[12] and a fluorescent gelator (BHMC). The galactose ensures Gal-BHMC to selectively bind with asialoglycoprotein receptor (ASGP-R) over-expressed on HCC cells membranes because of their high affinity and rapid internalization rate,[13-14] which could be examined by the fluorescent emission of Gal-

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Electronic Supplementary Information (ESI) available: X-ray crystallographic data, experimental procedures, supplemental figures, synthesis and characterization of all new compounds. See DOI: 10.1039/x0xx00000x

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DOI: 10.1039/C6CC05707A

BHMC inside cells under light excitation. The carbamate bond can be efficiently cleaved to precisely release BHMC gelators by UV light, since light offers the merits of relatively high precision spatial, temporal, and dose control by varying irradiation parameters, such as wavelength, intensity, and time.[16a] After cleavage, BHMC can self-assemble into nanofibers inside the cells and the nanofibers exhibit critical damage on cellular function and in turn induce HCC cell death (Scheme 1b). The study develops a methodology to design muti-functional nano-materials for the purpose of targeted and efficient cancer therapy by releasing the assembled gelators under external stimuli. Herein, UV light will be firstly explored for confirming the feasibility of the methodology since it is readily achieved.

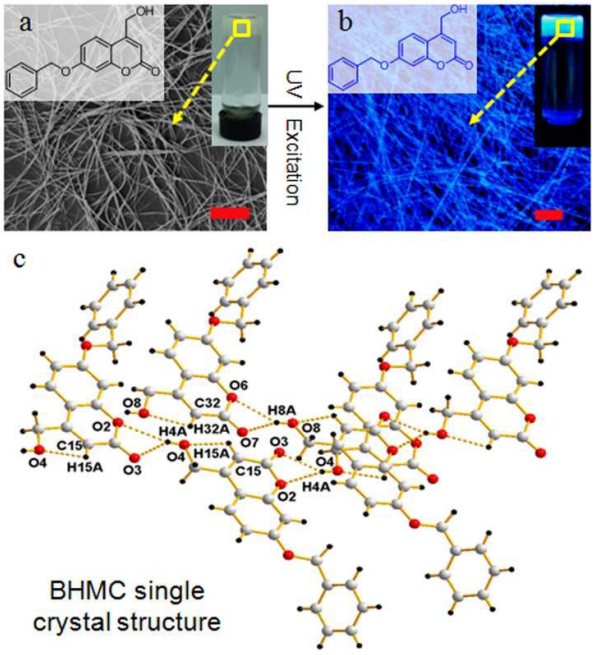

Fig. 1 a) SEM and b) fluorescent image of nanofibrous structures of freeze dried hydrogel BHMC, scale bars represent 50 and 100 μm, respectively. Insets are photographs of BHMC hydrogels before a) and after b) UV irradiation. c) Packing mode of BHMC stabilized by hydrogen bonds in single crystal state. (Gray C, black H, red O).

The precursor Gal-BHMC and hydrogelator BHMC were synthesized in several steps starting from 1,3-benzenediol (Scheme S1), respectively. The chemical structures of all the new compounds were confirmed by ^{1}H NMR, ^{13}C NMR, and HRMS (Figure S1-S21). To prove the self-assembly ability of BHMC, the powder of BHMC was initially dissolved in dimethyl sulfoxide (DMSO) and water was then added to trigger the gelation (Inverted vial in Figure 1a) with final DMSO concentration of 8% (a well-known method to make hydrogels).[15] To gain insight into the morphologies of hydrogel BHMC**,** the freeze dried thin layer of xerogels was studied by scanning electron microscopy (SEM) and Transmission Electron Microscopy (TEM). The well-defined nanofibers were observed with the thickness of ~10-200 nm and the length in the range of hundreds of micrometers (Figure 1a and Figure S22). Especially, the blue emission of hydrogel could be visualized under UV light due to fluorescent property of coumarin derivative (Inverted vial in Figure 1b).[16] The entangled nanofibrous structures with blue emission were further observed by fluorescence optical microscopy (Figure 1b). In addition, the critical gelation concentration (CGC) and critical aggregation concentration (CAC) of BHMC are 8 mM and 120 μM, respectively (Figure S23 and S24).

To investigate the self-assembly mechanism of BHMC, the colourless single crystal of BHMC was prepared by slow solvent evaporation method from n-hexane/tetra-hydrofuran solution and characterized by single crystal X-ray diffraction (SC-XRD).[17] Detailed crystallographic data were summarized in Table S1A, S1B, and S1C. BHMC crystallizes in the monoclinic space group P21/n with two molecules in the asymmetric unit (Z'=2), containing 8 independent molecules in one unit cell (Z=8) (Figure S25). The crystal structure and packing diagram of BHMC suggested that the main driving forces of the self-assembly were from two types of intermolecular hydrogen bonds (O-H···O) (Figure 1c). Two O atoms of lactone in coumarin ring were found to form hydrogen bonds with a hydrogen of benzyl alcohol (O4-H4A···O2, O4-H4A···O3, O8-H8A···O6, O8-H8A···O7), which have the bond length of 2.54 Å (H4A···O2), 2.21 Å (H4A···O3), 2.45 Å (H8A···O6), 2.14 Å (H8A···O7), respectively. In addition, two intramolecular hydrogen bonds of C15-H15A···O4 and C32-H32A···O8 could be also formed to further increase the stability of self-assembly with the bond length of 2.34 Å (H15A···O4), 2.33 Å (H32A···O8), respectively. Structure analysis proved that there were no π-π stacking (Cg-Cg Distances＞4.5 Å, Table S1D) between the hydrogelators. Followed by the frequently occurred hydrogen bonds between BHMC, they could self-assemble into three dimensional (3D) crystal networks. Moreover, powder X-ray diffraction (PXRD) was further investigated and it proved that PXRD patterns were essentially superimposable for both single crystal and xerogel states of BHMC (Figure S26), implying the same self-assembly mechanism for two states.[18]

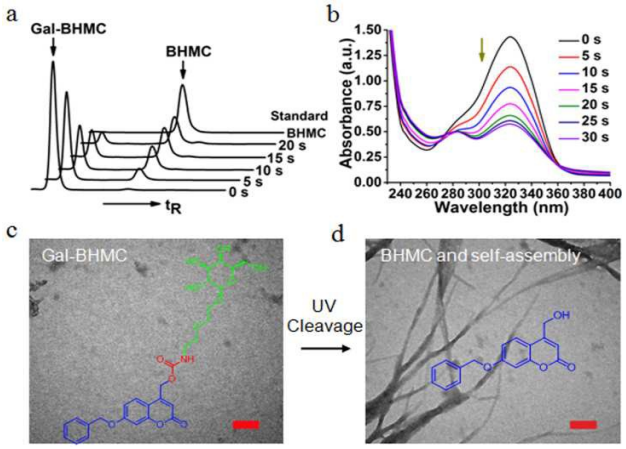

Fig. 2 a) HPLC and b) UV-Vis absorption traces for the photolysis of Gal-BHMC (2 x 10^{-5} M) in PBS solution. TEM images of the cryo-dried Gal-BHMC solution (500 μM) c) before and d) after UV irradiation for 30 s. Scale bar: 100 nm. Insert is the conversion of precursor Gal-BHMC to gelator BHMC after UV cleavage.

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The cleavage of precursors Gal-BHMC into hydrogelators BHMC in PBS solution was explored under UV light (Scheme S2). HPLC and UV-Vis absorption were used to assess the photolytic rate under mercury lamp irradiation at ~320 nm. This wavelength ascribes to the characteristic absorption band of coumarin rings from Gal-BHMC (Figure S27).[12b] By HPLC, the photocleavage of Gal-BHMC was precisely dependent on irradiation time and intensity, the photolytic processes were effectively progressing with irradiation (Figure 2a and S28). The photolytic consumption of Gal-BHMC followed a single exponential decay and the photolytic amount could reach ~90% after 20 s irradiation (100 mW/cm^2). In good agreement with HPLC analysis, UV-Vis absorption intensity of Gal-BHMC continually decreased at the wavelength of 324 nm with irradiation time from 0 to 30 s (Figure 2b). Here, there were no detectable side products during the photolysis for Gal-BHMC, implying the efficient and controllable cleavage of Gal-BHMC precursors under UV light irradiation.

After the cleavage of Gal-BHMC into BHMC hydrogelators, the ability to self-assemble into nanofibers for the cleaved BHMC was studied by Transmission Electron Microscopy (TEM). No any nanofibers were detected for Gal-BHMC (500 μM) before UV light irradiation (Figure 2c). While, the formed nanofibers were observed after irradiating Gal-BHMC for 30 s by UV light (Figure 2d). The size of the nanofibers was ~10-20 nm and length was hundreds nanometers, suggesting the successful cleavage of the precursor and self-assembly for the cleaved BHMC. The stability of Gal-BHMC in PBS solution was checked in the absence of light by detecting the remaining rate of the precursor after 24 h incubation at 37 °C (Table S2). The high remaining rate (97.38%) confirmed that Gal-BHMC was sufficiently stable from hydrolysis and suitable for cell-related study *in vitro*.

For validating the efficiently targeted cancer cells of Gal-BHMC (Figure 3a), HepG2 cells (a type of HCC cell lines) with high ASGP-R expression level and Hela cells (as control) with little expression of ASGP-R were selected. Due to the specific interaction between galactose and asialoglycoprotein, the selectively targeted HepG2 cells and effective endocytosis inside cells was directly proved by fluorescent images under UV light because of fluorescent emission property of Gal-BHMC (Figure 3b). As control, scarce amount of fluorescent signal was detected for Hela cells due to less expression of ASGP-R (Figure S29). To further prove whether the endocytosis process was predominantly mediated by ASGP-R and galactoside interactions, HepG2 and Hela cells were pre-incubated with free galactose (a well-known inhibitor of the receptor) for 15 min by varying concentration from 0 to 200 mM and then incubated with Gal-BHMC (500 μM) for 2 h. Cell viability experiment suggested that the concentration of free galactose have little damage to cells (Figure S30). The distinct decrease of fluorescence intensity was detected for HepG2 cells and the decreased intensity was proportional to the free galactose concentration (Figure 3c and S31). All these ascribed to the pre-block of ASGP-R by the free galactose and the precursors had little chances to bind with ASGP-R. As expected, the fluorescence intensity didn't change so much for Hela cells owing to the lack of ASGP-R. These results demonstrated that cellular uptake of Gal-BHMC was through ASGP-R-mediated endocytosis in HepG2 cells.[19]

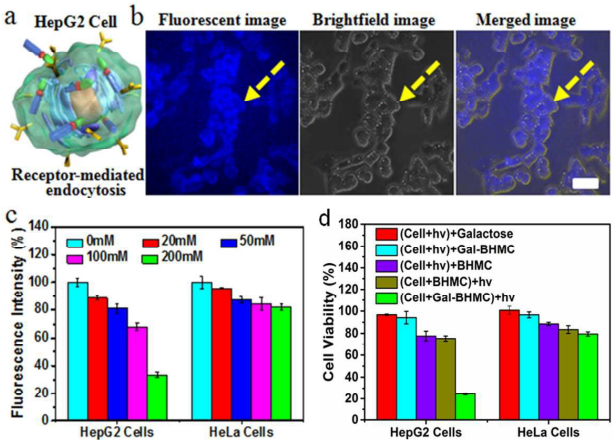

Fig. 3 a) Schematic demonstration of receptor-mediated endocytosis in HepG2 cells. b) Fluorescent, brightfield, and merged images of HepG2 cells with Gal-BHMC. Scale bar = 50 μm. c) Fluorescent intensity of HepG2 and HeLa cells pre-treated with free galactose and then incubated with Gal-BHMC. (d) The viability of HepG2 and HeLa cells detected by CCK-8 assay under different conditions by Gal-BHMC, BHMC, and galactose, respectively.

To evaluate the anticancer effects of intracellular self-assembly triggered by light, the viability of both HepG2 and HeLa cells was quantified by CCK-8 assay. UV light intensity of 40 mW/cm^2 was used in order to minimize the phototoxicity and the photolytic amount could reach over 70% after 30 s irradiation (Figure S28). The cells were firstly irradiated for 30 s and then incubated for 2 h with Gal-BHMC (500 μM), BHMC (500 μM), and galactose (500 μM), respectively. There was no significant cell death observed for both HepG2 and HeLa cells (Figure 3d), which implied that Gal-BHMC, BHMC, and galactose had no obvious toxicity to the cells. If the cells were firstly incubated for 2 h with BHMC (500 μM) and then irradiated for 30 s, which also showed little cytotoxicity for cells. However, the significant cell death was observed for HepG2 cells when they were firstly incubated with Gal-BHMC (500 μM) and then irradiated by UV light. This can be attributed to the conversion of Gal-BHMC into BHMC inside cells triggered by UV light and BHMC could further self-assemble into nanofibers, which may induced cell apoptosis through the promiscuous interactions between nanofibers and cytoskeletal proteins.[9] As comparison, there was no significant cell death observed for HeLa cells under the same experimental condition.

To further explore the formation of intracellular self-assembly of BHMC gelators, dead HepG2 cells were collected and broken by ultrasonication. TEM image of the cell lysate revealed the existence of entangled nanofibers (arrows in Figure 4) with ~10 nm in width and hundreds nanometers in length. The morphologies were very similar to the formed nanofibers by the cleaved BHMC in aqueous solution after UV irradiation (Figure 2d). It should be noted that the cell lysate of

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living HepG2 cells without Gal-BHMC incubation did not contain nanofibers by TEM (Figure S32). Furthermore, the HepG2 cells were harvested and lysed in dimethyl sulfoxide to dissolve the organic compounds from the cells for HPLC analysis. [4e] The intracellular concentration of BHMC and Gal-BHMC in HepG2 cells were also showed in Table S3. The result indicated that the intracellular concentration of BHMC (290 μM) in HepG2 cells was over the critical aggregation concentration (120 μM) and BHMC would self-assemble to form nanofibers. These results supported our hypothesis that the cell death was induced by the formation of intracellular self-assembly of BHMC cleaved from Gal-BHMC precursors.[6]

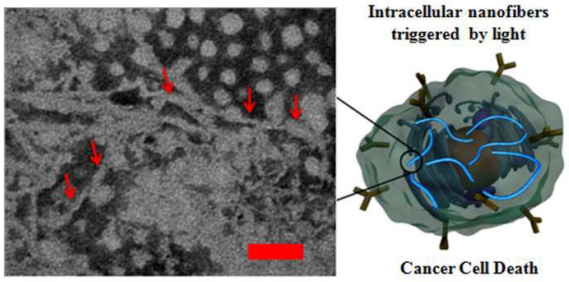

Fig. 4 TEM image of the lysate of dead HepG2 cells with the self-assembled nanofibers as dedicated by arrows Scale bars represent 100 nm.

In summary, a fluorescent and multi-functional gelator precursor with high photosensitivity was rationally designed, which can selectively target cancer cells through the receptor mediated interaction between galactose and ASGP-R over-expressed by cancer cells. Typically, the precursor can release hydrogelators inside cells under photo-irradiation, leading to intracellular self-assembly and subsequently inducing cell death. Especially, coumarin derivatives have the property of two-photon absorption, enabling to irradiate the precursors with near infrared light (*non-invasive wavelength*), which is still going on in our group. The study develops a methodology to design multi-functional nano-materials for the purpose of selective and efficient tumor therapeutics through precisely releasing the assembled gelators under external stimuli. The methodology can be further exploited in the design of similar systems to target different cancer cells by exchanging the galactose unit into other functional groups, which can specifically recognize the receptors over-expressed at cell membrane.

We thank the NSFC (51573092, 51273111, 51173105), the National Basic Research Program of China (973 Program 2012CB933803), the Shanghai Jiao Tong University (SJTU)−University of Michigan (UM) Collaborative Research Program, and the Program for Professor of Special Appointment (Eastern Scholar) at the Shanghai Institutions of Higher Learning.

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