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Tandem Molecular Self-Assembly in Liver Cancer Cells

Jie Zhan,* Yanbin Cai,† Shuangshuang He,* Ling Wang,* and Zhimou Yang‡

Abstract: We introduce in this study the tandem molecular self-assembly of a peptide derivative (compound 1) that is controlled by a combination of enzymatic and chemical reactions. In PBS, compound 1 self-assembles first into nanoparticles by phosphatase and then into nanofibers by glutathione. Liver cancer cells exhibit higher concentrations of both phosphatase and GSH than normal cells. Therefore, the tandem self-assembly of 1 also occurs in liver cancer cell lines HepG2 and QGY7703, in which compound 1 first forms nanoparticles around the cells and then forms nanofibers inside the cells. Compound 1 with this tandem self-assembly property exhibits a large ratio of cellular uptake and inhibition of cell viability between liver cancer cells and normal liver cells. We envision that using both extracellular and intracellular reactions to trigger tandem molecular self-assembly could lead to the development of supramolecular nanomaterials with improved performances in cancer diagnostics and therapy.

Self-assembly is a powerful tool for preparing supramolecular materials.1 Assisted by non-covalent interactions, small building blocks spontaneously and hierarchically assemble into functional materials.2 General approaches usually utilize chemical triggers, external energy, and solvent evaporation to trigger the self-assembly process, while biology takes advantage of biocatalysts to generate building blocks in a more controllable and efficient way. Inspired by nature, catalytic self-assembly has been widely explored to construct dynamic,3 dissipative4 and reversible5 nanostructures. These nanostructures not only offer a platform to understand the hierarchical self-assembly process in biological systems but also show promising applications in sensing,6 drug delivery,7 cell fate control,8 immune response manipulation,9 and regenerative medicine.10 However, research efforts mainly focus on using a single-step reaction catalyzed by an enzyme or using a small molecule to trigger the self-assembly process.11 Although kinetic control has shown pronounced influences on the outcome of single-step self-assembly,12 multi-step self-assembly could lead to more sophisticated and functional materials.

Using two reactions to control supramolecular self-assembly has attracted recent research interest. For example, the dephosphorylation/phosphorylation cycle catalyzed by the ALP/kinase switch has been applied to control the self-assembly of amphiphilic peptides,13 nanoparticles,13 and block copolymers.14 Xu group recently showed a control of molecular self-assembly by ALP and esterase that could selectively inhibit cancer cells with the down-regulation of esterase.15 Liang group also demonstrated a cell environment-differentiated molecular self-assembly with nanofiber-to-nanofiber transformation by an ALP-triggered dephosphorylation and a GSH-triggered condensation reaction.16 Xu and Maruyama’s groups have demonstrated extra-cellular17 and intracellular18 self-assembly trigged by enzymes, to selectively kill cancer cells. Inspired by reported pioneering studies of using two reactions to manipulate molecular self-assembly, we opt to combine Xu’s and Maruyama’s strategies and use the enzymatic reaction by extracellular phosphatase (ALP) and a chemical reaction by intracellular glutathione (GSH) to trigger the tandem self-assembly of a peptide derivative. The nanoparticles formed in situ around cells would show efficient cellular uptake through endocytosis compared with free molecules, and the transformed nanofibers within cells could be more efficiently retained by cells due to their larger size compared with nanoparticles. We therefore imagined that molecules with tandem molecular self-assembly could show an enhanced selectivity to cancer cells due to their higher concentrations of both ALP and GSH than normal cells.

To test our hypothesis, we designed and synthesized the molecule NBD-GFFPγ-ss-ERGD (1 in Figure 1A), which could be converted first to NBD-GFFY-ss-ERGD (2) by the extracellular ALP and then to NBD-GFFY-thiol (3) by intracellular GSH. Compound 1 could form a clear solution in phosphate buffer saline (PBS, pH = 7.4) solution at a concentration of 200 μM (0.03 wt%). Figure 1B showed that the clear solution remained after adding the enzyme of ALP (1 U/mL) at 37 °C for 12 h. The LC-MS trace indicated that more than 90% of 1 had been converted to 2 within 4h (Figures S12 and S13). We observed the formation of a yellowish gel after 10h after adding GSH (4 equiv. to 1) to the solution of 2. The LC-MS trace indicated that more than 85% of 2 had been converted within 10h (Figures S12 and S13). From the transmission electron microscopy (TEM) images, we observed nanoparticles in the solution of 2 with a diameter of 30-60 nm (Figure 1C) and nanofibers in the gel with a diameter of 10-25 nm (Figure 1D). We also monitored the fluorescence spectra of a solution of 1, a solution of 2, and a gel of 3. As shown in Figure S14, the solution of 1 showed a fluorescence peak centered at 550 nm with an intensity of 20834 a.u. After the addition of ALP to trigger the conversion from 1 to 2, the fluorescence intensity of solution 2 decreased to 8855 a.u. Upon addition of GSH, the fluorescence intensity further decreased to 2104 a.u. for gel 3. The decreases in the fluorescence intensity suggested the self-assembly of 2 in the solution and 3 in the gel. We then recorded the circular dichroism (CD) spectra of these samples (Figure S15). Compound 1 in solution adopted a random coil

[†] J. Zhan, S. He and Prof. L. Wang
State Key Laboratory of Medicinal Chemical Biology and College of Pharmacy, Nankai University, Tianjin 300071, China

[‡] J. Zhan, Y. Cai and Prof. Z. Yang
College of Life Sciences, Key Laboratory of Bioactive Materials, Ministry of Education, and Collaborative Innovation Center of Chemical Science and Engineering (Tianjin), Nankai University, Tianjin 300071, China, E-mail: yangzm@nankai.edu.cn

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Synthesis and characterization, CD, Fluorescence, Confocal images, Cellular uptake, Cell inhibition, Assays of ALP and GSH. These materials are available on the WWW under http://www.angewandte.org or from the author.
structure. The peptides in the nanoparticles of 2 and nanofibers of 3 adopted β-sheet-like conformations, which were indicated by a positive peak near 195 nm and a negative trough at 210-220 nm. These observations clearly indicated the tandem molecular self-assembly property of compound 1 controlled by ALP and GSH in PBS solution.

Figure 1. A) Chemical structures and schematic illustration of conversion from 1 to 2 by phosphatase (ALP) and then from 2 to 3 by glutathione (GSH), B) optical images of solution of 1 (200 μM, 0.03 wt%), solution of 2 formed by adding ALP (1 U/mL) to solution of 1, and gel of 3 formed by adding GSH (4 equiv.) to solution of 2, and TEM images of C) solution of 2 and D) gel of 3 (scale bars in C) and D) represent 500 nm.

Cancer cells expressed higher concentrations of extracellular ALP and intracellular GSH than normal cells, and the different environments between extra- and intra-cellular spaces of cancer cells might provide an ideal platform to explore the tandem self-assembly processes of 1. According to the World Cancer Report 2014 by WHO, primary liver cancer is the second leading cause of death from cancer (9%). We therefore first used HepG2 liver cancer cell line for the study. The blue fluorescence in the confocal laser scanning microscopy (CLSM) images was from Hoechst 33342, representing the nucleus of the cells. The yellow fluorescence belonged to the NBD-peptides. As shown in Figures 2A and S16A, we observed yellow fluorescent dots around HepG2 cells after incubating the cells with 1 (200 μM) for 0.5 h, and we observed yellow fluorescent fibers inside the cells at the 4h time point (Figures 2B and S16). The TEM images also revealed nanoparticles with diameters of 30-70 nm in the culture medium of HepG2 cells at the 0.5h time point (Figure 2D) and nanofibers with a diameter of approximately 6-25 nm in broken HepG2 cells at the 4h time point (Figure 2E). We observed a dense fiber network in QGY7703 cells at the 4h time point (Figures 2C and S16), which was also a liver cancer cell line. However, we only observed yellow fluorescent dots inside other cancer cell lines of HeLa, MCF-7, PC-3, and A549 cells (Figure S17), and we hardly observed yellow fluorescent dots on the cell membrane of the normal liver cell lines L02 and QSG7701, even at the 4h time point (Figure S18). We found that the six cancer cell lines expressed higher levels of extracellular ALP than the two normal cell lines (Figure S19), and the two liver cancer cell lines possessed higher concentrations of intracellular GSH than the other four cancer lines and two normal cell lines (Figure S19). These observations suggested that the tandem self-assembly of 1 in extra- and intracellular environments of liver cancer cells was due to the high concentrations of both ALP and GSH in liver cancer cells. Although intracellular self-assembly has been recently demonstrated as a powerful strategy for a spatiotemporal profile of enzyme activity,[6, 18] those self-assembled fluorescent probes did not show obvious fiber structures within the cells, while our results directly and clearly showed the formation of intracellular fibers in liver cancer cells. It was important to note that the distribution of nanofibers was different between the QGY7703 and HepG2 cells. It appeared that the nanofibers in the QGY7703 cells grew from the cell membranes, while those in the HepG2 cells grew from the nuclear membranes. The reasons for such differences would be studied in the near future.

We also prepared control compounds without the tandem self-assembly behavior, NBD-GFFY-ss-ERGD (2) in nanoparticles, NBD-GFFY-Thiol (3) in nanofibers, and NBD-GFFpY-GERGD (4) without the GSH responsive disulfide bond. We observed yellow fluorescent dots in the HepG2 cells when the cells were treated with these control compounds at the 4h time point (Figure S20). The yellow fluorescent dots colocalized well with the red fluorescence from Lyso-Tracker in HepG2 cells, which suggests that the nanoparticles of 2, nanofibers of 3 and compound 4 were mainly in the lysosomes at the 4h time point (Figure S20). At the same time, the yellow fluorescent fibers of 1 did not overlay well with the red fluorescence from Lyso-Tracker in the HepG2 cells. The yellow fluorescence from the nanofibers in the HepG2 cells did not overlay well with the red fluorescence from actin-Tracker or tubulin-Tracker (Figure S21) either, indicating that the nanofiber formation was not due to the sticking of our compound to the tubulin or actin filaments. Pretreatment with tripeptide RGD resulted in a very weak yellow fluorescence in the HepG2 cells with a following co-treatment of 1.
and tripeptide RGD for 4h (Figure S22), implying that nanoparticles were mainly taken up by cells through interacting with integrin.

We then studied the cellular uptake behavior of different compounds in different cells. We incubated cells with different compounds at a concentration of 200 μM for 4 h. The results in Figures 3A and S23 showed that for compound 1, HepG2 and QGY7703 cells exhibited higher cellular uptakes than the other four cancer cell lines and two normal cells. For the nanoparticles of 2 and compound 4, both liver cancer and normal cells showed similar cellular uptakes. For the nanofibers of 3, the two normal liver cells possessed higher cellular uptake than the liver cancer cell lines. If calculating the cellular uptake ratio between liver cancer cells and liver normal cells (Figure 3B), the value of HepG2/L02 and HepG2/QSG7701 for 1 was 5.58 and 4.46, respectively. This value was 0.71 and 0.98, respectively, for 2, 0.35 and 0.49, respectively, for 3 and 1.92 and 2.40, respectively, for 4. A similar trend was observed in the comparison of the cellular uptake value of different compounds between QGY7703 and the other two liver normal cells. These observations suggested that molecules with the tandem self-assembly property could be more efficiently taken up by liver cancer cells than by normal liver cells.

Extensive studies have demonstrated that extracellular and intracellular nanofiber formations might lead to cell death. We therefore compared the cell viability of both cancer and normal cells treated with different compounds (Figures S24-S26). The results in Figure S26 indicated that compound 1 showed the smallest ratio of relative cell viability between liver cancer cells and normal cells. For example, the ratio of cell viability at 200 μM was 0.23, 0.22, 0.43 and 0.42 for for HepG2/L02, HepG2/QSG7701, QGY7703/L02 and QGY7703/QSG7701, respectively. Nanoparticles of 2 also showed a moderate selectivity in inhibiting HepG2 and QGY7703 cells over L02 and QSG7701 cells. In contrast, nanofibers of 3 exhibited no difference in cell viability for both liver cancer and normal cells. Another control compound, NBD-GFFpY exhibited similar cell viability to several types of cancer cells (HepG2, QGY7703, HeLa, MCF-7, A549, PC-3, Figure S27), which was most likely due to their similar expression levels of ALP (Figure S19). NBD-GFFpY exhibited a small inhibition effect to QSG7701 and L02 cells at concentrations of 200 μM due to their low concentration of ALP. These observations clearly indicated an enhanced selectivity to killing liver cancer cells by tandem molecular self-assembly.

The diagram in Figure 3C illustrates the tandem self-assembly of 1 in liver cancer cells and explains the differences in the cellular uptake and cell viability inhibition capacity between liver cancer and normal cells. Compound 1 possessed highly negative charged phosphorylated and carboxylic acid groups and, therefore, showed poor cellular uptake to cells. Once it was converted to 2 by ALP, compound 2 would self-assemble into nanoparticles, which could stick to cell membranes via interacting with integrin and therefore be efficiently taken up by cancer cells through endocytosis. Once taken up, compound 2 could be further converted to 3 by intracellular GSH, resulting in nanoparticle to nanofiber transformation. The intracellular nanofibers might change the viscosity of cancer cells or interact with important proteins within cells, thus leading to cancer cell death.

Nanoparticle-to-nanofiber transformation in tumor tissues or cancer cells had been demonstrated as a promising strategy for better retention of nanomedicines in tumors. Although nanoparticles showed an enhanced permeability and retention effect on tumors, they had limited tumor penetration abilities. Since free molecules could freely diffuse in tissues, molecules with a tandem self-assembly property might show better performance in cancer diagnostics and treatments. In this study, we clearly demonstrated in vitro and in liver cancer cells a tandem molecular self-assembly of a peptide derivative that was controlled by a combination of enzymatic and chemical reactions. Facilitated by the higher concentrations of both ALP and GSH in liver cancer cells than normal cells, the molecules with a tandem self-assembly property exhibited an enhanced selectivity to cancer cells. Our study might provide a useful strategy to design supramolecular nanomaterials for liver cancer diagnostics and therapy.

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References


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Tandem Molecular Self-Assembly in Liver Cancer Cells

Compound 1 self-assembles first into nanoparticles by phosphatase and then into nanofibers by glutathione, which works well in buffer solution and in liver cancer cells due to the higher concentrations of both phosphatase and GSH in liver cancer. Compound 1, having such a tandem self-assembly property, exhibits big differences in cellular uptake and cell viability between liver cancer cells and normal cells.