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PAPER

pH responsive smart carrier of [60] fullerene with 6-amino-cyclodextrin inclusion complex for photodynamic therapy†

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A new pH responsive smart carrier of C_{60} was prepared with 6-amino-γ-cyclodextrin (ACD) for photodynamic therapy. C_{60} was rapidly released from its inclusion complex with ACD at pH 6.7, due to electrostatic repulsion between ammonium groups, followed by a rapid C_{60} release at slightly acidic cancer cell surfaces.

Photodynamic therapy (PDT) can be regarded as a patient-friendly anticancer therapy because it does not involve ionizing radiation, which allows it to be used repeatedly without cumulative long-term complications since it does not appear to target DNA. Recent studies on PDT with first generation photosensitizers have shown some efficacy in the treatment of a variety of malignant and premalignant conditions, including head- and neck-cancers,[1] lung-cancer,[2,3] mesothelioma,[4] Barrett's esophagus,[5,6] prostate[7] and brain tumors.[8,9] Among the various types of first generation photosensitizers, [60] fullerene (C_{60}) is an emerging material for photodynamic therapy because photoexcited C_{60} generates activated oxygen species, resulting in efficient extinction of the vicinal tissues.[10–15] However, for realistic applications its insolubility in water is limiting. To overcome this shortcoming, many attempts at improving the water solubility of C_{60} have been developed with a variety of chemical[16,17] and physical modifications.[18–21] In this context, inclusion complexes of C_{60} with γ-cylodextrin (γ-CD) have received a lot of attention as a facile solubilization technique, without elaborate chemical treatments. However, from a practical viewpoint of the smart delivery of C_{60} to the specific cancer cell, extremely stable hydrophobic interactions between C_{60} and γ-CD inhibits uptake into cancer cells. Therefore, a rational design leading to optimal stability and controlled release of the γ-CD/C_{60} inclusion complex considering external stimuli, such as pH, temperature, and light, is highly desired for practical photodynamic therapy applications. Recently, several approaches targeting the extracellular pH of cancer cells have been developed using water soluble polymers with covalently attached hydrophobic anticancer drugs as side chains.[22,23] Here a slightly acidic extracellular pH (pH = 6.4–7.2) was utilized as an external stimulus for chemical cleavage of the covalent linkage between the polymer backbone and the anticancer moieties. However, these methods require dexterous molecular designs to provide adequate chemical responsiveness at specific pH conditions. Therefore, a smart carrier that rapidly releases C_{60} in response to the extracellular pH of cancer cells remains challenging. In this study, we demonstrated for the first time that a cyclodextrin (CD) derivative bearing primary amino groups at the primary face acts as a smart carrier of non-substituted hydrophobic C_{60} to the slightly acidic cancer cells. Consequently, the efficient uptake of C_{60} exhibited excellent photodynamic activity against cancer cells.

As a pH responsive moiety, a primary amine was monofacially introduced into the 6-positions of CD (ACD) (Fig. 1a).[24,25] Here we expected that the amino groups at the 6-positions would not interfere in the inclusion of C_{60} with ACD under neutral pH conditions because C_{60} is only accessible from the bottom face of ACD to form the inclusion complex (ACD/C_{60}). Only when the amino groups of ACD were protonated did the upper face expand due to electrostatic repulsion between the protonated amino groups, followed by shrinkage of the bottom face. In conjunction with the deformation of the ACD rings, the included C_{60} is rapidly squeezed from the inclusion complex to the vicinal cancer cells (Fig. 1b and c). In this study, ACD/C_{60} was prepared by a solid-state mechanochemical reaction as described elsewhere (see ESI†).[26] Briefly, pristine C_{60} was vigorously shaken with ACD powder by high-speed vibration milling at 1800 rpm for 20 min. The resulting mixture was dispersed in water, and then the insoluble portion was removed by centrifugation. The obtained supernatants were kept as a stock solution. The ACD/C_{60} solution was colored light-violet, and did not exhibit precipitation

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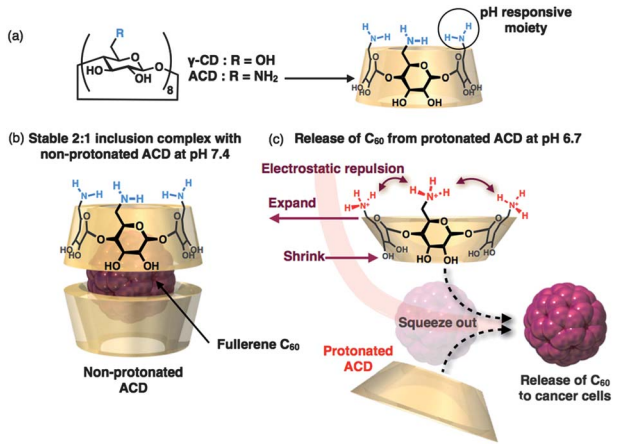

Fig. 1 (a) Chemical structure of 6-amino-γ-cyclodextrin (ACD), and (b) schematic representation of 2 : 1 inclusion complex of ACD and C_{60}. (c) Proposed mechanism of release from the smart carrier of C_{60} with ACD inclusion complex in response to the protonation of amino groups at extracellular cancer cell pH (pH 6.4–7.2).

over a week under neutral pH conditions. This suggests that C_{60} is homogeneously isolated in ACD/C_{60} under neutral pH conditions. The stoichiometric ratio of the host–guest complex could not be directly estimated by conventional titration because high-speed vibration milling is a solid-state reaction process. However, from the evidence from UV-vis (ultraviolet-visible) absorption data and a recent molecular simulation study,[27] we proposed that an isolated ACD/C_{60} complex forms a 2 : 1 complex (Fig. 1). By changing the pH from 9 to 4, the color of the ACD/C_{60} solution rapidly changed from light-violet to brownish yellow at a critical pH of 6.7, indicating that C_{60} formed a colloidal aggregation (Fig. 2a). From dynamic light scattering analysis, the average diameter of the C_{60} colloidal aggregation was estimated to be *ca.* 20 nm (Fig. S1, ESI†). Here the C_{60} colloidal aggregations should be stabilized by the protonated ACD under lower pH conditions. On the other hand, the light-violet solution of γ-CD/C_{60} remained unchanged in the pH region between 4 and 9, due to the absence of the pH responsive moiety in γ-CD over this pH region (Fig. 2b and S2, ESI†). The significant changes in the solution color were further elucidated by UV-vis spectroscopy. Thus, the ACD/C_{60} solution exhibited an intense UV signal at 332 nm, originating from isolated C_{60} (Fig. 2c).[28] Upon changing the pH from 8.9 to 4.2, this peak became broad and red-shifted to 338 nm. In addition, a broad signal around 450 nm appeared. Similar spectral changes are often seen in the aggregation of C_{60}.[29]

Unlike the γ-CD/C_{60} inclusion complex, ACD/C_{60} exhibited a unique release of C_{60} in response to subtle pH changes slightly lower than neutral conditions. We performed a pH titration of ACD/C_{60} with 1 N HCl. The pH of ACD/C_{60} gradually changed without a sudden step upon the addition of 1 N HCl (Fig. 2d, blue solid line). Therefore, the amino groups of ACD appear to adopt multistep protonation, probably due to steric effects among neighboring amino groups. In spite of the gradual changes in the protonation of amino groups, C_{60} was rapidly released at a critical pH of 6.7 (Fig. 2d, red solid line). To address the unique release behavior, computational studies with

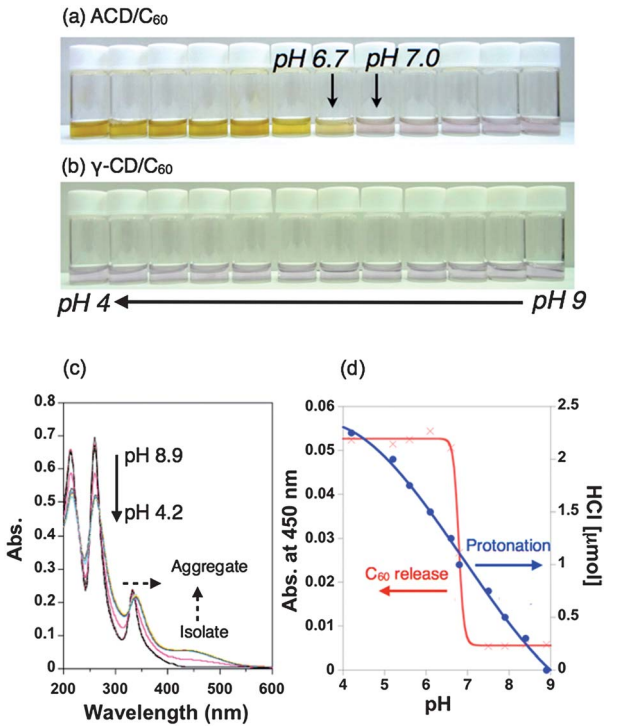

Fig. 2 Photographs of (a) ACD/C_{60} and (b) γ-CD/C_{60} at pH ranges of 4 to 9. (c) Changes in UV-vis spectra of ACD/C_{60} upon changing the pH from 4.2 to 8.9. (d) Changes in UV-vis absorption of ACD/C_{60} at 450 nm (red) and amount of 1 N HCl (blue) required to adjust the final pH from 4.2 to 8.9.

molecular mechanics calculations were performed using PCFF force fields (Materials Studio 4.0, Accelrys Inc., San Diego, CA) (Fig. 3). To simplify the calculations, a 1 : 1 inclusion complex was adapted as a model for an initial configuration, instead of a 2 : 1 inclusion complex. Non-, half-, and fully-protonated ACDs were used as transition models for ACD gradually becoming

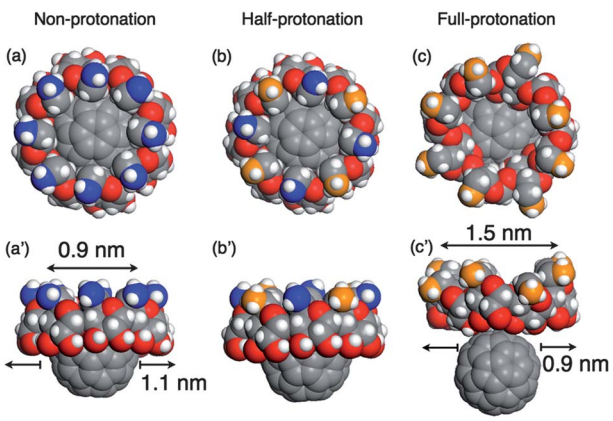

Fig. 3 Proposed structures of ACD/C_{60} complexes. (a) Top view of C_{60} and non-protonated ACD, (b) C_{60} and half-protonated ACD, and (c) C_{60} and fully-protonated ACD. (a′), (b′), and (c′) represent the side views of the corresponding models. The atom colors gray, white, red, blue and orange represent C, H, O, N and N^+ respectively. To simplify the calculation, 1 : 1 inclusion complexes of C_{60} and ACD were employed. PCFF was used as a force field (Materials Studio 4.0).

protonated. In the half-protonated ACD, amino groups are alternately protonated. Consequently, non-protonated ACD could tightly accommodate C_{60} in ACD, due to hydrophobic interactions between C_{60} and the inner cavity of ACD (Fig. 3a and a′). Interestingly, half-protonated ACD did not show significant changes in conformation, and C_{60} remained stably located in the ACD (Fig. 3b and b′). This is because hydrophobic interactions between C_{60} and ACD overcame the deformation energy derived from electrostatic repulsion between protonated amino groups. Eventually, when the amino groups were fully protonated, the upper face of ACD increased from 0.9 nm in diameter to 1.5 nm. Conversely, the bottom face of ACD decreased from 1.1 nm in diameter to 0.9 nm (Fig. 3c and c′). Responding to the deformation, C_{60} was immediately squeezed from the inner cavity of the ACD, followed by the formation of the colloidal aggregations.

Finally, we demonstrated the *in vitro* photodynamic activity of the ACD/C_{60} complex using human cervical cancer HeLa cells (see ESI†). ACD/C_{60} solution was added to the cell cultures. Here, the solutions were adjusted to be pH 7.4 and pH 6.4 with PBS buffer, corresponding to the extracellular pH of normal cells and cancer cells, respectively. Successively, the cell cultures were exposed to visible light (400–500 nm) for 30 min. After continuous light irradiation, cell viability was evaluated using a WST-8 assay (Cell Counting Kit-8: Dojindo Laboratories Co., Kumamoto, Japan). As a reference, γ-CD/C_{60} was used as a non-responsive C_{60} carrier at the extracellular pH of cancer cells. It has been reported that the photodynamic activity of γ-CD/C_{60} is negligibly low, although it does not show dark toxicity.[30] This is because C_{60} could not be released from the stable γ-CD/C_{60}, leading to an inhibition of C_{60} uptake into the cancer cells.[31,32]

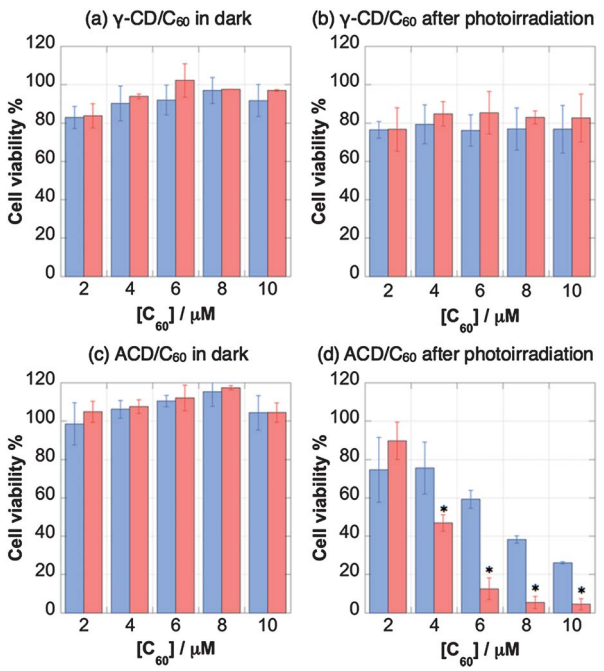

Fig. 4 Cell viability of HeLa cells treated with γ-CD/C_{60} ((a) in the dark; (b) after photoirradiation) and ACD/C_{60} ((c) in the dark; (d) after photoirradiation). The viability was tested at pH 7.4 (blue columns) and pH 6.4 (red columns). *$p < 0.05$ as compared with the activities at pH 7.4.

Indeed, γ-CD/C_{60} did not express photodynamic activity at either pH 6.4 or 7.4 (Fig. 4b), and exhibited a lack of dark toxicity under the same pH conditions (Fig. 4a). On the other hand, ACD/C_{60} showed excellent photodynamic activity. Thus, cell viability significantly decreased with an increase of ACD/C_{60} concentration from 2 to 10 μM (Fig. 4d). Similar to γ-CD/C_{60}, an apparent dark toxicity was not evident (Fig. 4c). Furthermore, the cell viability at pH 6.4 was greater than that at pH 7.4. Judging from the pH titration of ACD/C_{60}, the protonated ACD readily released C_{60} at pH 6.4. Following that, the released C_{60} could be taken up into the cancer cells within 30 min. Here, C_{60} is thought to be adsorbed to the hydrophobic parts of the cell membrane, as is often seen in artificial membranes.[14,15,18,20,21,33,34] Thus, active oxygen species were produced by photoexcited C_{60} incorporated into the cancer cell membrane, and then unsaturated phospholipids or membrane proteins were oxidized, leading to efficient cell death. Here it is noteworthy that cell viability at pH 7.4 upon visible light irradiation was significantly decreased down to 30%, although this effect is less than that at pH 6.4. It is well known that the extracellular pH of the human cancer cell is acidified at approximately pH 5–6. Thus, even under the neutral buffer condition (pH 7.4), the extracellular pH vicinal HeLa cell appears to be rather acidic. Therefore, ACD/C_{60} is thought to release C_{60} nearby the HeLa cell surface, followed by uptake of a certain amount of C_{60} into the cell surface (Fig. S3, ESI†). Consequently, significant photodynamic activity was expressed even at pH 7.4. On the other hand, in the case of pH 6.4, the ACD/C_{60} forms colloidal aggregations surrounded by protonated ACDs with an average diameter of 20 nm in the bulk solution. Therefore, a large amount of C_{60} aggregations in the bulk solution were taken up into the HeLa cells, probably through endocytosis, followed by the expression of the more efficient photodynamic activity (Fig. 4). To further confirm the proposed uptake mechanism of C_{60}, the direct observation of C_{60} in HeLa cells was demonstrated with TEM (transmission electron microscopy) (see ESI†). Consequently, after 30 min incubation of ACD/C_{60} in the presence of HeLa cells at pH 6.4, it was revealed that C_{60} existed both in the cell membrane (Fig. 5a) and within the intracellular vehicle (Fig. 5b). These results strongly supported the conclusions that (i) direct insertion to the cell membrane and (ii) uptake into the cell through endocytosis were simultaneously carried out (Fig. 6).

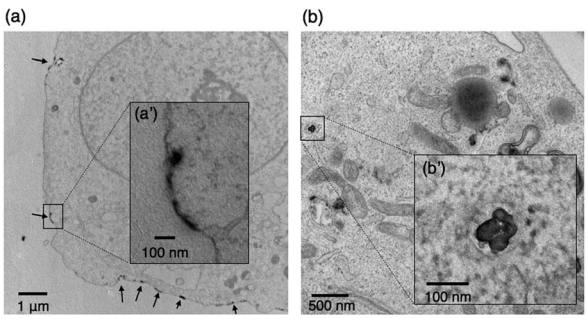

Fig. 5 TEM images of ACD/C_{60} localization in a HeLa cell (a) without staining and (b) stained with uranyl acetate/lead citrate. (a′) Enlarged image of the square in (a). (b′) Enlarged image of the square in (b). C_{60} was located in the cell membranes (indicated by arrows in (a)) and within the intracellular vehicle (the square in (b)).

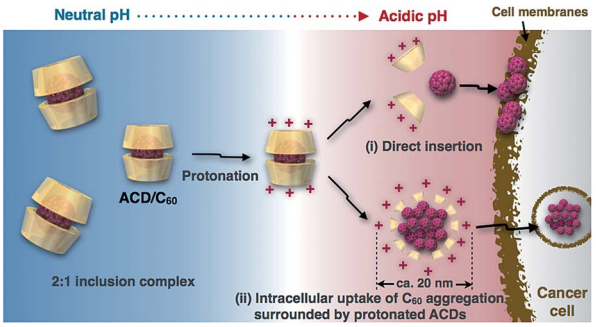

Fig. 6 Schematic representation of efficient cellular uptake mechanisms; (i) direct insertion to the cell membranes and (ii) intracellular uptake of C_{60} aggregation surrounded by protonated ACDs.

In conclusion, we demonstrated that a cyclodextrin derivative with monofacially introduced amino groups at the 6-positions (ACD) successfully acted as a smart carrier for non-substituted C_{60} to cancer cells, resulting in significant photodynamic activity under slightly acidic conditions, as a mimic of extracellular cancer cell conditions. The rational design of a sensitive pH responsive carrier was achieved by adjusting the stability of the ACD/C_{60} inclusion complex. In this system, the protonation of the amine groups acted as a trigger for deformation of the ACD rings, leading to the efficient release of C_{60} in response to subtle changes in pH around neutral. We also succeeded in demonstrating *in vitro* photodynamic activity of ACD/C_{60}. C_{60} was rapidly taken up into the cancer cells at pH 6.4, resulting in significant photodynamic activity under the pH conditions of extracellular cancer cells. The present simple but effective pH responsive smart carrier with amino-cyclodextrin could be applicable for a variety of drug deliveries in physiological environments by optimizing the ring size of ACD and/or the amine derivatives with different basicity.

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