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Accumulation of supramolecular nanoparticles self-assembled from a bola-shaped cytidylic acid-appended fluorescein dye in cell nuclei†

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We examined the cellular uptake of the nanoparticles self-assembled from a bola-shaped cytidylic acid-appended fluorescein derivative (C-FLU-C). The accumulation of fluorescence in the Caco-2 cell nucleus was observed mainly after the plateau phase of cell growth, indicating that C-FLU-C permeated the nuclear envelope without nuclear-localizing tags.

The development of techniques for intracellular delivery of materials has attracted the attention of many researchers owing to the potential application of these techniques in biomedical and pharmaceutical uses such as bioanalysis or *in vivo* imaging.[1–6] Particularly, the delivery of materials to the nucleus is considered to be an important technique because that is where genetic information is stored; however, most nanomaterials reported so far remain trapped in the cytoplasm after penetrating the cell membrane. One of the main reasons for this is that the nuclear envelope acts as a barrier to protect the nucleus. Pores in the nuclear envelope regulate the exchange of molecules between the nucleus and the cytoplasm by restricting the physical size of molecules that can enter the nucleus by passive diffusion and signal-mediated transport mechanisms to around 9 and 39 nm, respectively.[7] To overcome this problem, nanomaterials conjugated with peptide-based nuclear-localizing signal (NLS) tags have been developed that successfully translocate into cell nuclei.[8–11] Interestingly, Cheng *et al.* have reported the accumulation of PEGylated SWCNTs in cell nuclei without modification with NLS tags.[12] Although the translocation of nanomaterials without NLS tags into the nucleus must provide new insight into the use of nanomaterials for the targeting of cell nuclei, there still remain unclear factors such as design of nanomaterials or uptake mechanisms into cell nuclei.

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It is widely accepted that a bottom-up approach based on self-assembling building blocks is a good strategy for the production of well-defined, nanostructured materials.[13] In aqueous solution, amphiphilic molecules self-assemble into nanostructures such as micelles, vesicles, fibers, and tubes, and these nanostructures are expected to be used in many fields as, for example, sensor membranes,[14] hydrogelators,[15] or templates for inorganic materials.[16–19] In particular, nanostructures formed from self-assembling amphiphiles have attracted a lot of attention as promising vehicles for intracellular delivery of materials because of their affinity with the cell membrane.[3,20,21] Previously, we studied the self-assembly of nucleotide-appended bolaamphiphiles, which are amphiphilic molecules with a hydrophilic nucleotide moiety at both ends of a long hydrophobic chain, and we found that these bolaamphiphiles form stable nanostructures, such as nanosheets and helical and no helical nanofibers, in aqueous solution.[22–25] Cytidylic acid-appended bolaamphiphiles spontaneously form stable nanoparticles with a relatively homogeneous diameter and size distribution of $\leq$100 nm in aqueous solutions due to weaker stacking interactions between cytosine moieties.[23] In the present study, we synthesized fluorescent nanoparticles self-assembled from a cytidylic acid-appended fluorescent amphiphilic molecule, **C-FLU-C**, to perform cellular uptake experiments of nanomaterials. We observed that the self-assembling fluorescent nanoparticles were taken up into cells and that cell-dependent accumulation occurred in the nuclei of two mammalian (Caco-2 and BALB/3T3) cell lines.

We designed and synthesized cytidylic acid-appended fluorescein (bis[5-(3′-cytidylyloxy)pentyloxy]fluorescein; **C-FLU-C**) according to procedures described previously (Fig. 1a and Fig. S1, details in ESI†). Owing to the hydrophilic cytidylic acid moieties at each end of the fluorescein molecule, **C-FLU-C** showed good solubility in water and aqueous buffer solutions and spontaneously formed fluorescent nanoparticles. We used transmission electron microscopy, atomic force microscopy and size distribution measurement to verify the morphology of the nanoparticles, and we confirmed that the **C-FLU-C** nanoparticles in aqueous solution had a mean diameter of 70 nm and a relatively narrow size distribution (Fig. 1b; Fig. S2 and S3, ESI†). In addition, TEM images (Fig. 1b and Fig. S2, ESI†)

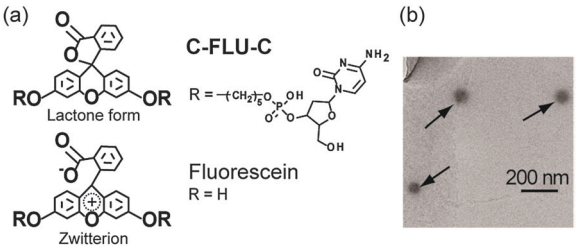

Fig. 1 (a) Structure of cytidylic acid-appended fluorescein (**C-FLU-C**). (b) Transmission electron microscopic image of self-assembled **C-FLU-C** nanoparticles. Arrows indicate nanoparticles.

displayed no internal space in the nanoparticles, indicating that the nanoparticles are not vesicles as observed in the self-assembly from cytidylic acid-appended bolaamphiphiles.[23] Emission spectra showed maxima at around λ 530 nm for both **C-FLU-C** and fluorescein in Tris-HCl aqueous buffer solution (pH 8, ex = 480 nm, Fig. S4, ESI†). The fluorescein with a lactone structure does not show fluorescence and absorbance in the visible region;[26] therefore, the fluorescence of **C-FLU-C** should originate from the zwitterionic structure of the fluorescein skeleton (Fig. 1a, lower). The yellow color of the aqueous solution containing the self-assembled **C-FLU-C** is explainable on the basis of the formation of zwitterionic structure of the fluorescein moiety.[27]

Fluorescence from **C-FLU-C** was localized in the nuclei upon incubation with Caco-2 cells (human colon carcinoma cell line). Flow cytometric analysis (Fig. 2a and b) showed that fluorescence intensity increased after **C-FLU-C** was incubated with the Caco-2 or BALB/3T3 (contact-inhibited semi-normal cell line: mouse embryo cell line) cell line, implying that **C-FLU-C** either entered the cells or was adsorbed on the cell surface. The uptake efficiency[28] curve of **C-FLU-C** into Caco-2 cells dramatically increased up to day 5, after which it gradually increased until it was $>80\%$ at day 14 of culture (Fig. 2c, ●). However, in BALB/3T3 cells, uptake efficiency remained constant after day 5 at approximately 55% (Fig. 2c, ▲). We did not observe any intracellular uptake of **C-FLU-C** by the Caco-2 cell line cultured at 8 °C to suppress cellular activity. This observation suggests that **C-FLU-C** enters Caco-2 cells *via* an energy-dependent mechanism. Growth curves of Caco-2 and BALB/3T3 cells in the presence or absence of **C-FLU-C** gave similar standard sigmoidal curves that became confluent at day 6 (Fig. S5, ESI†). **C-FLU-C** was therefore not toxic to either cell line. Together the results for cellular uptake efficiency and cell growth show that the uptake

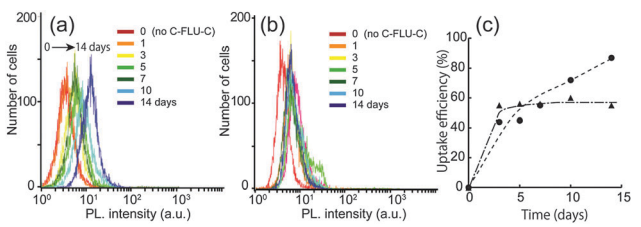

Fig. 2 Flow cytometric analysis of (a) Caco-2 cells + **C-FLU-C** and (b) BALB/3T3 cells + **C-FLU-C**. (c) Time-dependent uptake efficiency of **C-FLU-C** into Caco-2 (●) and BALB/3T3 (▲) cells.

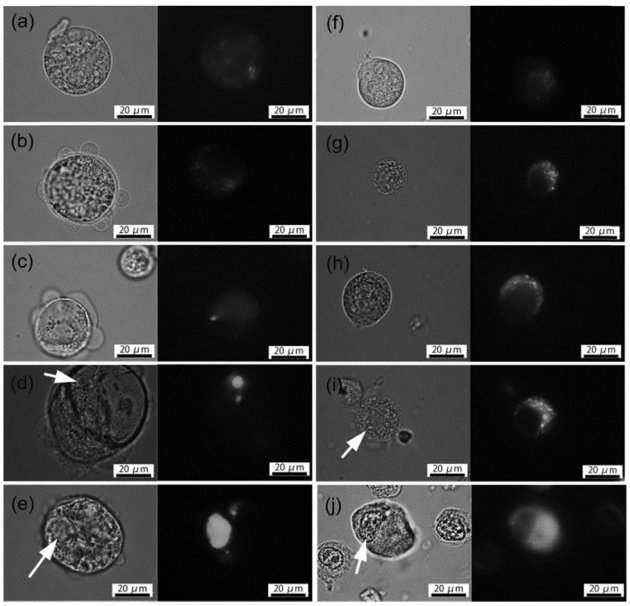

Fig. 3 Light (left) and fluorescence (right) microscopic images of Caco-2 cells + **C-FLU-C** cultured for (a) 1 day, (b) 4 days, (c) 5 days, (d) 7 days, and (e) 12 days, and of BALB/3T3 + **C-FLU-C** cultured for (f) 2 days, (g) 5 days, (h) 6 days, (i) 7 days, and (j) 13 days. Arrows indicate nuclei.

efficiency of **C-FLU-C** nanoparticles into BALB/3T3 cells increased most during cell division, whereas that of Caco-2 cells increased most after cell growth reached confluence after 6 days of culture. This observation suggests that the uptake of **C-FLU-C** nanoparticles by Caco-2 cells depends on mechanisms involved in cell division or on other mechanisms such as those involved in endocytosis. Fluorescence microscopic analysis was used to confirm the intracellular uptake of **C-FLU-C**. A green fluorescence signal was observed in the cytoplasm of both cell lines (Fig. 3). We further confirmed the entry of **C-FLU-C** into Caco-2 cells by means of laser-scanning confocal microscopy of the cell cytoplasm (Fig. S6, ESI†). Interestingly, Caco-2 cells cultured in the presence of **C-FLU-C** for 7 days showed the translocation of fluorescence into the cell nuclei (Fig. 3d), and the fluorescence signal remained after 12 days of culture (Fig. 3e); whereas, no fluorescence was observed in the nuclei of BALB/3T3 cells (Fig. 3f–j).

To further investigate the accumulation of **C-FLU-C** in the nuclei of Caco-2 cells, we carried out cell culture experiments using both cell lines and fluorescein. Although we obtained images of fluorescence in the cytoplasm after culturing the Caco-2 or BALB/3T3 cell line with fluorescein for 11 to 13 days (Fig. 4), we did not observe the accumulation of fluorescence in the nuclei of either cell line. This result suggests that the accumulation of **C-FLU-C** in the cell nucleus is specific to Caco-2 cells and that the cytidylic acid moiety in **C-FLU-C** is crucial for its transportation into the nucleus. In fact, after colcemid treatment (to arrest cell growth at the M phase, during which the nuclear envelope disassembles), **C-FLU-C** was adsorbed on the chromatin of both Caco-2 and BALB/3T3 cells (Fig. S7, ESI†). This result strongly indicates that the nuclear envelope prevents the transportation of **C-FLU-C** into the nucleus of BALB/3T3 cells. Although large molecules may enter the nucleus

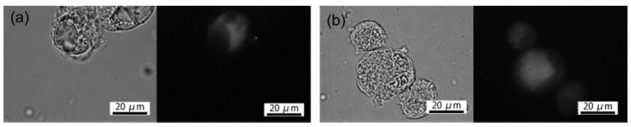

Fig. 4 Light (left) and fluorescence (right) microscopic images of Caco-2 cells + fluorescein cultured for (a) 11 days and of BALB/3T3 + fluorescein cultured for (b) 13 days.

during mitosis when the nuclear envelope breaks down, they are generally excluded when the nucleus reassembles after mitosis.[29] Unlike most large molecules, Cheng *et al.* reported that PEGylated SWCNTs that are not conjugated to nuclear-localizing signal tags localize not only in the nuclei of parent HeLa cells (human epithelial carcinoma cell line) but also in the nuclei of daughter HeLa cells.[12] Cheng *et al.* suggest that PEGylated SWCNTs tether to proteins or RNAs that are reimported into the nucleus after mitosis.[12] In the present study, marked accumulation of fluorescence from **C-FLU-C** in Caco-2 cell nuclei was seen after the cell growth curve reached a plateau (after around 7 days). This observation differs from that seen with PEGylated SWCNTs,[12] indicating that in Caco-2 cells, **C-FLU-C** did not enter the nucleus during mitosis, but instead permeated the cell envelope after the nucleus was reassembled and then translocated into the nucleus. Nuclear pores should not allow the transportation of **C-FLU-C** nanoparticles (mean diameter, 70 nm) into the nucleus, because the nanoparticles are too large.[7] Therefore, we hypothesize that **C-FLU-C** nanoparticles attach to or near nuclear pores in Caco-2 cells, dissociate, and are then transported into the nucleus as **C-FLU-C** molecules or clusters. Interestingly, the adenovirus capsid (d = 80 nm) dissociates and imports the capsid contents (DNA) into the cell nucleus after docking at nuclear pores.[30] We hypothesize that the mechanism responsible for the translocation of **C-FLU-C** into Caco-2 cell nuclei could be similar to that responsible for the transport of the adenovirus capsid contents.

In summary, fluorescein, which is widely used for the imaging and labeling of biomolecules in cells,[31] was used to synthesize the molecular building block **C-FLU-C**, which self-assembles into nanoparticles. **C-FLU-C** nanoparticles were efficiently taken up by Caco-2 and BALB/3T3 cells without the need for additional physical or chemical treatments. Uptake of **C-FLU-C** nanoparticles did not affect the cell cycle under experimental conditions. We demonstrated the cell-dependent transfer of **C-FLU-C** into the nuclei of Caco-2 cells. Together these findings provide insights into the cell-selective translocation of materials into the cell nucleus that will be helpful in the development of biomedical and pharmaceutical applications using nanoparticles such as cell-selective drug delivery and imaging.

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