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NanoPARCEL: a method for controlling cellular behavior with external light†

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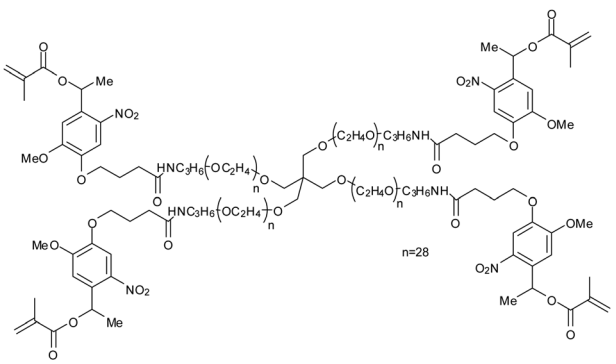
We developed a simple preparation procedure for the protein encapsulated nanoparticle and used the nanoparticle for spatiotemporal activity control of various proteins. We succeeded in the local protein activation within cells by light using the nanoparticle.

The biological functions of proteins are precisely controlled so as to sustain life. The latest proteome analysis methods permit profiling of all intracellular proteins expressed during a sampling period and have revealed many new important roles of proteins.[1,2] However, understanding cellular processes (*e.g.*, proliferation, differentiation, migration, and death) induced by changes in intracellular protein levels simply by analyzing the levels at a given time is difficult. The ability to reproduce cellular processes by controlling protein levels can be expected to improve our understanding of the mechanisms of cellular processes. Noninvasive methods for controlling intracellular protein levels, such as the use of knockout mice, small interfering RNA, and light-activated protein, have been reported,[3–10] but a general method for spatiotemporal control of protein activity within cells is lacking. Here, we describe a general, noninvasive method for spatiotemporal control of the activity of various proteins in cells.

We previously reported a method for photocontrol of protein activity using a photodegradable hydrogel (Protein Activation and Release from Cage by External Light; PARCEL).[11,12] Proteins are encapsulated in a cross-linked gel network with a mesh size smaller than the protein diameter; the encapsulated protein is inactive because the network restricts protein interactions with other compounds. The encapsulated protein is released under physiological conditions by stimulation with external light, whereupon protein activity is restored. This method can be used for various proteins because no chemical modification of the proteins is required. However, PARCEL cannot be used to control protein activity within cells, because the gels are too large (millimeter scale) for introduction into cells and because the gels contain acrylamide, a suspected neurotoxin and carcinogen.

In this study, we succeeded controlling protein activity within cells by biocompatible 150 nm nanoparticles, which have been successfully used as biomolecule carriers.[13–17] Three different nanoparticles containing different proteins were prepared and used to carry the proteins into two different cell lines. Protein release and activation were controlled by irradiation at 365 and 405 nm.[18] Cell damage at these wavelengths is negligible,[19] and the timing, location, and intensity of the light could be controlled. We also experimented with controlling cellular processes by changing the timing and location of protein activation within living cells. We refer to this method as nanoscale PARCEL (nanoPARCEL).

First, we synthesized a novel four-arm photocleavable linker PEG-Photo-MA (Scheme 1) from polyethylene glycol and a photocleavable unit. Each arm bears a photocleavable group and a terminal methacryloyl unit.[20] The linker was cleaved by UV irradiation (356 and 405 nm).[18] Polymerization of the linker afforded an acrylamide-free gel. Surprisingly, we obtained 150 nm nanoparticles by vortexing the photocleavable linker for 20 min at 4 °C. The size distribution of the protein-containing nanoparticles was narrow (average diameter ~150 nm, as indicated by transmission electron microscopy (TEM); Fig. 1 and dynamic light scattering (DLS); Fig. S1, ESI†). Although trypsin concentration did not affect the nanoparticle size, the ammonium persulfate (APS) concentration regulated the nanoparticle size



PEG-Photo-MA

Scheme 1 Chemical structure of PEG-Photo-MA.

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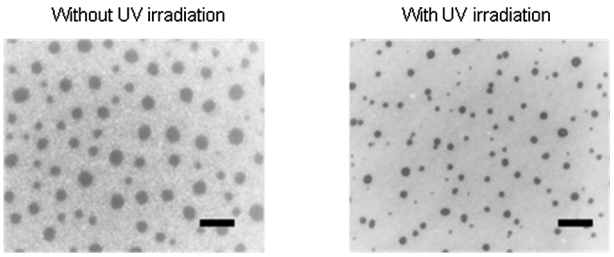

Fig. 1 Transmission electron micrographs of photodegradable nanoparticle-encapsulated trypsin with and without UV irradiation. All scale bars are 200 nm.

(Fig. S2, ESI†). No method for nanoparticle preparation at high APS concentrations and low temperature has previously been reported.[21,22] The high APS concentration favored seed formation; many seeds grew simultaneously. Nanoparticle growth stopped when the monomer supply became insufficient. At high APS concentrations, growth stopped quickly, because many nanoparticles grew simultaneously.

TEM (Fig. 1 and Fig. S3, ESI†) and DLS (Fig. S1, ESI†) results suggested that the UV irradiation (365 nm 20 s 0.57 W cm^{-2}) reduce the size of the nanoparticle (<60 nm). It is supposed that the nanoparticles had collapsed owing to cleavage of the gel network.

To evaluate the nanoparticles as protein carriers *in vitro*, we encapsulated trypsin within the nanoparticles and evaluated its stability and release efficiency by comparing trypsin activities using BODIPY-casein, a fluorogenic substrate. Trypsin activity was negligible without UV irradiation but strong with UV irradiation (Fig. 2 and Fig. S4, ESI†). The activity of the released trypsin was about 20% when compared with that of the original trypsin (Fig. S5, ESI†). It is suggested that irradiation cleaved the gel network, and the released trypsin hydrolyzed BODIPY-casein. The reproducibility of trypsin activity after irradiation was an acceptable value (RSD = 15.5%) for cell assay.

To verify the effectiveness of these nanoparticles as protein containers within cells, we microinjected a mixture of dextran-Texas Red (an indicator of injection) and nanoparticles containing BODIPY-casein into living Cos-7 monkey kidney cells. When BODIPY-casein is hydrolyzed by intercellular enzymes, it fluoresces (data not shown). After injection, half the cells were irradiated with UV light for 10 s (0.25 W cm^{-2}), and the remaining half were not subjected to any external stimulus. Many cells were alive and unchanged in morphology after injection and irradiation. In confocal laser scanning microscopy (CLSM) images of the cells obtained 2 h after irradiation (Fig. 3), the cytosol appeared red, indicating distribution of the injected solution throughout the cytosol (right panels). No green fluorescence was observed before irradiation, when the labeled casein was encapsulated within the nanoparticles (upper left panel). In contrast, green fluorescence was observed after the labeled casein was released and then digested by intracellular proteins (lower left panel). This result indicates that the nanoparticles were stable in the cells before irradiation and that the encapsulated protein was released by irradiation.

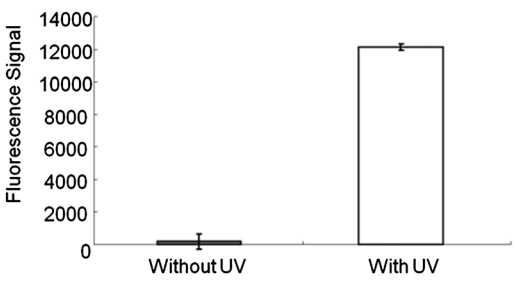

Fig. 2 Regulation of trypsin activity by means of UV irradiation of trypsin-containing nanoparticles *in vitro*. All samples were measured in quintuplet, and error bars indicate standard deviations.

Control of regulatory protein activation in cells can be expected to permit regulation of various cellular processes. To achieve such regulation, we injected HuH-7 cells with a mixture of dextran-Texas Red and nanoparticles containing activated caspase-3, which plays a role in cell death. Then we irradiated half the cells with UV light for 10 s. In the merged phase contrast microscopy (PCM) and CLSM images of the cells before irradiation and 24 h later (Fig. S6, ESI†), the fluorescence of Texas Red was visible just after injection and even after 24 h. Although the irradiated noninjected cells remained undamaged, all the irradiated cells with nanoparticles disappeared within 24 h, indicating that all the cells were dead and that the released caspase-3 induced cell death within 24 h. To confirm the release of the active caspase-3, we used the cells which were treated with a fluorogenic substrate ($CR(DEVD)_2$). The red signal of the $CR(DEVD)_2$ was observed from the cells on the process of the cell death (Fig. S7, ESI†).

Finally, we demonstrated the regulation of cellular processes (cell death) by releasing activated caspase-3 at a specific location in a living cell. Nanoparticles containing activated caspase-3 were introduced into a cell that had been transfected with an end-binding 1 (EB1)-green fluorescence protein (GFP) plasmid to label the microtubules with GFP in advance for visualizing dynamic changes in the cytoskeleton (Fig. S8, ESI†). When a spot of the cell (red circle, Fig. 4) was irradiated with laser, protein was released and activated only at the irradiated spot, and the released protein then diffused throughout the cell. The movie in ESI† shows two cells: one was with

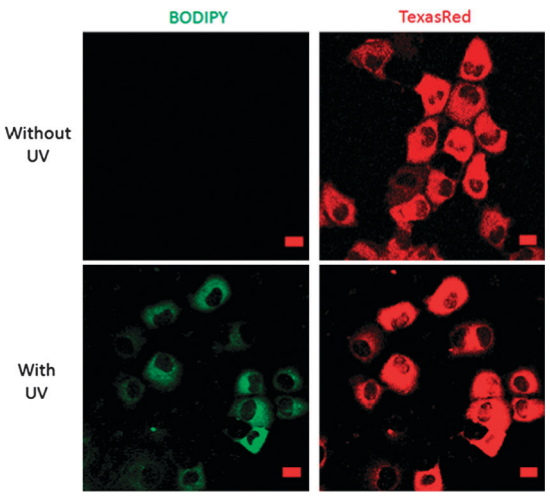

Fig. 3 Photo induced release of BODIPY-casein within cells. CLSM images of Cos-7 cells injected with nanoparticle-encapsulated BODIPY-casein with and without UV observed at 2 h after irradiation. Green, hydrolyzed BODIPY casein; red, dextran-Texas Red. All scale bars are 20 μm.

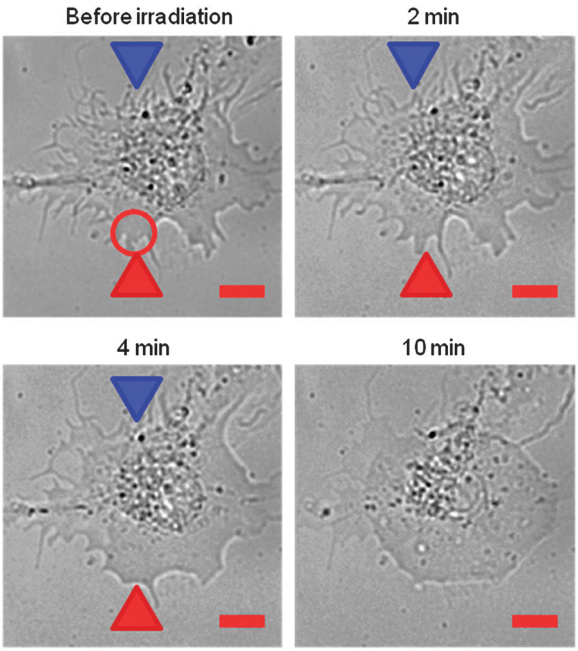

Fig. 4 Local activation of caspase-3 by focused light. PCM images show the progress of cell death induced by focused light: before irradiation and 2, 4, and 10 min after irradiation. The red circle indicates the irradiation point. All scale bars are 20 μm.

no nanoparticles without irradiation (upper side of the movie), whereas the other contained nanoparticles with irradiation and underwent cell death induced by the focused light (lower side of the movie). Fig. 4 and Fig. S8 (ESI†) show magnified images of this cell. Although the lamellipodia at the cell edge distant from the irradiation point did not change (Fig. 4, blue arrows, upper edge of cell), the lamellipodia at the irradiated edge retracted at about 2 min after irradiation (red arrows, lower edge of cell). Four minutes after irradiation, the cell started to expand at the irradiated edge while the lamellipodia at the opposite edge maintained their original shape. The irradiation point coincided with the point at which the lamellipodia change started, suggesting that the change was initiated by local activation of caspase-3. After approximately 10 min, the cell ruptured. The morphological change in the cell nucleus (Fig. 4) and dilution of the GFP fluorescence indicated cell death (Fig. S8, ESI†). Although the time required for cell death depended on the cell, all the cells died within 20 min. Cell death was not observed when the same experiment was performed with nanoparticles that did not contain activated caspase-3 (Fig. S9, ESI†). It is supposed that the nanoparticle and its degradates were less invasive to cells.

It was reported that protein function is changed by the activation area and timing, for example, caspases have various other functions in addition to inducing apoptosis, and many researchers are interested in the local cellular functions of caspases.[23] Investigation of local protein activity within cells is difficult because methods, other than nanoPARCEL, for spatiotemporal control of the activity of a variety of proteins within cells are limited.

Although we could control the local activity of proteins, protein diffusion was fast, and the protein concentration gradient disappeared after about 10 min. To circumvent this problem, activation of local protein and monitoring of cellular processes should be nearly simultaneous. The nanoPARCEL method could be used to activate proteins at a specific area of a cell, and the triggered cellular processes could be observed immediately after protein activation using a commonly used microscopy technique.

In summary, proteins were effectively released and activated within cells upon irradiation of protein-containing photodegradable nanoparticles with external light. Because chemical modification is not necessary for activity control, nanoPARCEL should be amenable to a variety of proteins. This low invasive method for spatiotemporal control of protein activity in cells should permit the regulation of cellular processes, and we expect that research on protein function will be accelerated by application of the method and that it will be a powerful tool for characterization of complex cellular mechanisms.

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