Supramolecular Hydrogels

DOI: 10.1002/anie.201404158

Two-Photon-Responsive Supramolecular Hydrogel for Controlling Materials Motion in Micrometer Space**

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Abstract: *Spatiotemporal control of fluidity inside a soft matrix by external stimuli allows real-time manipulation of nano/micromaterials. In this study, we report a two-photon-responsive peptide-based supramolecular hydrogel, the fluidity of which was dramatically controlled with high spatial resolution (10 μm × 10 μm × 10 μm). The off–on switching of the Brownian motion of nanobeads and chemotaxis of bacteria by two-photon excitation was successfully demonstrated.*

Supramolecular hydrogels[1] formed by the self-assembly of small molecules are promising biomaterials for numerous applications, such as cellular scaffolds,[2] controlled drug release,[3] and biosensing.[4] Compared to conventional polymer gels, supramolecular hydrogels are anticipated to exhibit unique functions, such as generating fluidic nanofiber networks[5] and dynamic or flexible stimuli-responsive properties.[6] It is now recognized that precise control of the gel structure and functions is crucial for the construction of sophisticated soft biomaterials comprising supramolecular hydrogels not only to facilitate understanding of the impact of the surrounding environment on a unique biological function, but also for manipulating various biological phenomena.[7]

Many stimuli-responsive supramolecular hydrogels[6] have been developed to date. Light is an attractive stimulus for such systems because of its contactless mode and high spatiotemporal resolution. Indeed, several research groups have reported photoresponsive supramolecular hydrogels based on a photoisomerization reaction or photo-click chemistry.[8] However, these simple systems in which UV light is used for irradiation, show cytotoxicity without careful dose adjustment. In regard to biomaterials application, two-photon responsiveness is superior to one-photon responsiveness because of its higher biocompatibility. A few distinctive chromophores are able to absorb two less energetic photons simultaneously upon irradiation with intense laser pulses[9] to generate the same excited state as that with one-photon excitation. This allowed light with a twofold longer wavelength to be utilized (typically, near-infrared (NIR) region), which is more appropriate for fabricating hydrogels with lower cytotoxicity. As another advantage, three-dimensional (3D) fabrication inside the gel matrix can be performed with high spatial resolution by the two-photon process, because the two-photon excitation event occurs only at a focal point, which depends on the numerical aperture of the lens, the wavelength of the light, and the refractive index of the materials. In this context, it was recently reported that two-photon-responsive polymer gels,[10] for example, hydrogels consisting of poly(ethylene glycol) cross-linked with a photolabile *o*-nitrobenzyl group,[10a,b] can be employed as cell culture matrices for controlling extracellular microenvironment.[10a] Although the inside fluidity was not quantitatively examined in detail, the polymers remaining after photodegradation are presumed to remain in the irradiated space. By contrast, two-photon-responsive supramolecular hydrogels have not yet been developed, despite these being expected to show a drastic change in fluidity because all the photogenerated residues are small molecules. We describe herein the design of a peptide-based supramolecular hydrogel capable of exhibiting gel–sol transition upon two-photon excitation, which enables the creation of 3D fluidic micrometer-sized spaces inside the gel with high spatial resolution and with a high fluidity equivalent to an aqueous solution. We also successfully demonstrated local control over the Brownian motion of nanobeads and the regulation of the chemotaxis of living bacteria in an off–on manner, without inactivation of the biological processes inside the two-photon-responsive supramolecular hydrogel matrix.

We recently established a semirational design strategy for various stimuli-responsive peptide-based supramolecular hydrogelators.[11] For example, by incorporating a photostimuli-responsive 7-bromo-hydroxycoumarin-4-yl-methoxycarbonyl (Bhcmoc) protecting group at the N-terminus of a dipeptide (FF; F: phenylalanine), a supramolecular gel was obtained, which showed a gel–sol transition on (one)

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[**] Prof. K. Akiyoshi, Dr. S. Mukai, Dr. H. Aoki (Kyoto University), and Prof. S. Yokoyama (Kyushu University), Mission of the Center for Meso-Bio Single-Molecule Imaging (CeMI) are thanked for technical help in microscopy observations. Dr. Y. Takaoka (Kyoto University, Hamachi Lab) and Prof. K. Urayama (Kyoto Institute of Technology) are acknowledged for help with TEM observations and rheological measurements, respectively. T.Y. acknowledges JSPS Research Fellowships for Young Scientists. This work was partly supported by CREST (Japan Science and Technology Agency).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201404158.

phototriggered cleavage of the Bhcmoc moiety (Figure 1). We sought to explore two-photon-responsive hydrogels based on this scaffold. Since the Bhcmoc-FF moiety does not form a hydrogel at a physiological pH value, probably because of

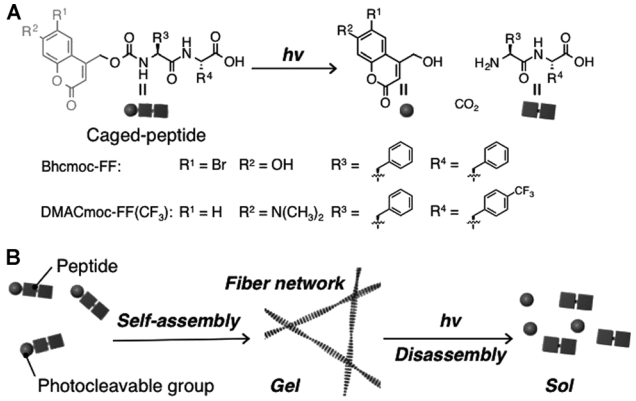

Figure 1. A) Photoinduced cleavage of a caged-peptide gelator. B) Schematic representation of the self-assembly of a caged-peptide gelator to form a supramolecular hydrogel and its photoresponsive gel–sol transition.

deprotonation of the hydroxy group of Bhcmoc, we decided to replace the Bhcmoc group at the N-terminus of peptides with a dimethylaminocoumarin-4-yl-methoxycarbonyl[12] (DMACmoc) group, a typical two-photon absorption dye. A small library of DMACmoc-modified peptides was constructed by varying the peptide sequence (see Figure S1A in the Supporting Information) and gelator screening was conducted at pH 7.4. By the tube-inversion method, we found four gelators that form transparent hydrogels that should be appropriate for efficient photoabsorption with minimization of photodiffraction in neutral aqueous conditions (pH 7.4; see Figure S1 in the Supporting Information). The critical gelation concentrations (CGCs) of each gelator were evaluated (see Figure S1B in the Supporting Information). DMACmoc-FF(CF_3) showed the lowest CGC (0.050 wt%), and was thus mainly used for further studies.

The nanostructure of the dried DMACmoc-FF(CF_3) gel was analyzed by transmission electron microscopy (TEM). A fibrous structure with diameters of 20–30 nm and lengths of several micrometers was observed (see Figure S2A in the Supporting Information). Observation of the DMACmoc-FF(CF_3) gel stained with a cationic fluorescence dye (DEAC-gua:[4b] 10 μM; see Figure S2B in the Supporting Information) by confocal laser scanning microscopy (CLSM) clearly revealed the presence of fiber network in a semiwet state (Figure S2C). The circular dichroism (CD) spectrum suggested the formation of a β-sheet-like secondary structure and a chiral arrangement of the aromatic side chains of the peptide and DMAC chromophore in the fibrous aggregates.[13] The rheological data of the DMACmoc-FF(CF_3) hydrogel showed the typical viscoelastic properties of a hydrogel consisting of fiber networks (see Figure S3 in the Supporting Information).[14] To evaluate the mesh size of the DMACmoc-FF(CF_3) gel, we then encapsulated fluorescent nanobeads and examined their Brownian motion.[15] Random movement (Brownian motion) of beads with a diameter of 250 nm in the DMACmoc-FF(CF_3) gel (0.075 wt%) was observed (see Figure S4A in the Supporting Information). In contrast, the Brownian motion of beads having a diameter of 500 nm stopped as a result of entrapment by the entangled gel fiber mesh (Figure S4B). These results indicate that the DMACmoc-FF(CF_3) hydrogel (0.075 wt%) formed a nanomesh with void spaces between 250 nm and 500 nm. The relationship between the Brownian motion of different sized beads and gelator concentrations was evaluated in detail (see Figure S4C in the Supporting Information).

To confirm the photoresponsive nature of the DMACmoc-FF(CF_3) gel, we firstly irradiated the gel (0.080 wt%) with one-photon excitation by a stand-alone Hg lamp (360 nm, 49 mW, 5 min), which caused a macroscopic gel–sol transition (see Figure S5A in the Supporting Information). HPLC analysis of the photoirradiated sample revealed a decrease in the amount of DMACmoc-FF(CF_3) and the concurrent increase in the amount of the cleaved product H-FF(CF_3) in an irradiation time dependent manner (see Figure S6 in the Supporting Information). These results indicate that the cleavage of the DMACmoc moiety occurred by one-photon excitation. The gel to sol transition occurred after 56% of the gelator had decomposed, which was in good agreement with the CGC value of this gelator. It is clear that removal of the DMACmoc group by one-photon (360 nm) excitation disturbs the delicate balance of the molecular interactions in the self-assembled nanofiber, which induces the gel–sol transition. The photoinduced gel-sol transition process can also be tracked by the disappearance of the fibrous morphology by CLSM (see Figure S5B in the Supporting Information).

We next examined the local gel–sol transition inside the DMACmoc-FF(CF_3) gel upon two-photon excitation (Figure 2A). A two-photon laser scanning microscope was utilized both for observing the supramolecular fiber and for fabricating the supramolecular hydrogel at different powers,

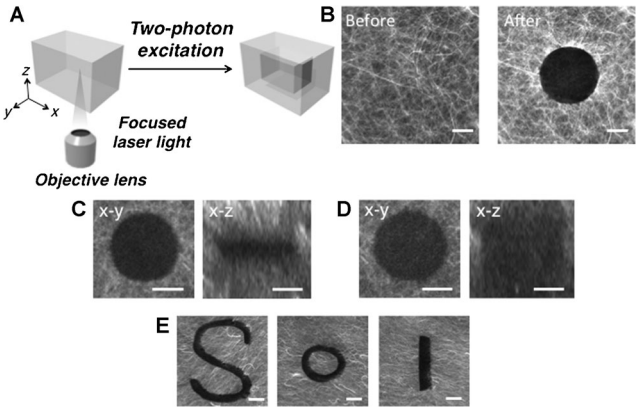

Figure 2. A) Schematic representation of the photofabrication of the DMACmoc-FF(CF_3) gel by two-photon excitation. B) CLSM images of the DMACmoc-FF(CF_3) gel (0.1 wt%) stained with DEAC-gua (10 μM) before and after two-photon irradiation (740 nm, 2 s). Scale bar: 10 μm. C,D) CLSM images of the *x*-*y* and *x*-*z* cross-section of the DMACmoc-FF(CF_3) gel fabricated by C) two-photon or D) one-photon excitation. Scale bar: 10 μm. E) Alphabetical letters patterned by two-photon excitation. Scale bar: 20 μm.

typically, 0.4% for the fiber observations and 10% for the gel fabrications (near-IR (NIR) pulse laser at 740 nm, 3 W, 140 fs). When the DMACmoc-FF(CF_3) gel (0.10 wt%) stained with DEAC-gua (10 μM) was irradiated with the NIR laser (10%), the entangled fibers disappeared in the irradiated area after a total irradiation time of only 2 s (Figure 2B). It is noteworthy that the inside of the DMACmoc-FF(CF_3) gel was three-dimensionally fabricated by two-photon excitation with 8 μm spatial resolution in the *z*-axis direction (Figure 2C (right)). In contrast, the spatial resolution in the *z*-axis by one-photon fabrication (UV laser at 405 nm, 30 mW, 30%, 2 s)) was rather broad, that is 25 μm (Figure 2D; right). The two-photon fabrication of the DMACmoc-FF(CF_3) gel was apparently three times more precise than the one-photon fabrication. This difference is attributed to the difficulty of the one-photon excitation to perfectly prevent the formation of the excited state in the space through which the light passes. Furthermore, the current response time is short (2 s) enough to fabricate more complex patterns, such as alphabetical letters, by scanning NIR laser light (Figure 2E).

We subsequently sought to perform a remote photomodulation of the Brownian motion of nanobeads inside the gel matrix through the focal gel-to-sol transition upon two-photon irradiation. The DMACmoc-FF(CF_3) gel encapsulating 500 nm diameter nanobeads was treated with NIR light (740 nm, 3 W, 10%, 2 s). Before photoirradiation, the locations of all the beads at 0 s overlapped well with those after 10 s by CLSM observation (Figure 3A, see also Movie S1 in the Supporting Information), which indicates that the Brownian motion of the beads stopped through entrapment by the nanomesh of the gel fibers. After NIR irradiation, the locations of the beads did not merge in the irradiated area, whereas the overlap still remained in the unirradiated area, which revealed that the Brownian motion restarted only on photoirradiation. To evaluate the speed of the Brownian motion of the beads inside the gel, the motion of the beads was traced every 0.49 s and the migration distance was plotted against time (see Figure S7 in the Supporting Information). The speed in the irradiated area was determined to be $(1.6 \pm 0.6)\ \mu m\,s^{-1}$. By contrast, the bead speed was approximately 25-fold slower ($0.06 \pm 0.04\ \mu m\,s^{-1}$) in the unirradiated area (Figure 3B). Assuming a two-dimensional Brownian motion according to the Einstein–Smoluchowski relation, the mean-square displacements were analyzed as a function of time and using the Stokes–Einstein Equation. This allowed us to estimate the local viscosity (η) in the irradiated area to be 2.8×10^{-3} Pa s. Remarkably, this is almost comparable to the viscosity of pure water (8.9×10^{-4} Pa s),[16] which suggests that highly fluidic focal space was created inside the hydrogel matrix by the two-photon stimuli. These results indicate that the fluidity increased (or the viscosity decreased) only in the irradiated focal space as the fiber disappeared (see above), as a result of the two-photon responsive gel–sol transition.

Given that the diameter of bacteria (250–1000 nm) is almost comparable to these beads, it might be reasonable to expect that a bacteria strain (*E. coli* RP437[17]) would be encapsulated in the DMACmoc-FF(CF_3) gel (0.075 wt%). Observation by CLSM confirmed that chemotaxis of the bacteria stained with SYTO9 did not occur in the DMACmoc-FF(CF_3) gel (see Figure S8 in the Supporting Information), thus indicating that the nanomesh of the DMACmoc-FF(CF_3) gel is strong enough to hinder bacterial chemotaxis. Interestingly, we observed that bacteria in the area irradiated with NIR light (740 nm, 3 W, 10%, 2 s) developed a motile state and moved in the limited microspace (Figure 4, see also

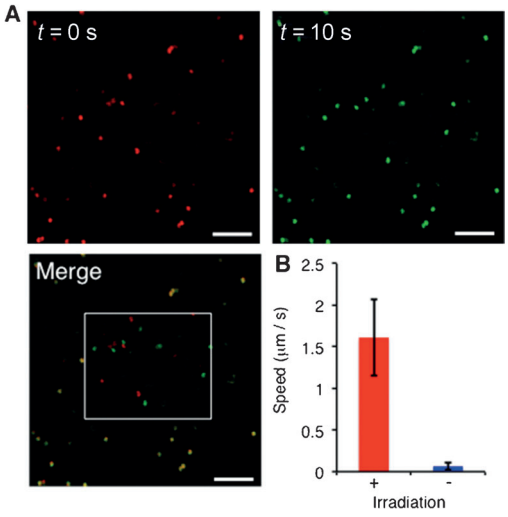

Figure 3. Controlling the local fluidity of the DMACmoc-FF(CF_3) gel (0.075 wt%) by two-photon excitation, evaluated by the Brownian motion of nanobeads. A) Time-lapse CLSM images of the fluorescent nanobeads (500 nm). The inset rectangle shown in the "Merge" panel was irradiated by focused laser light (740 nm). The fluorescence images were converted into pseudocolors (red: $t = 0$ s, green: $t = 10$ s) and merged. Scale bar: 5 μm. (see also Movie S1 in the Supporting Information). B) Comparison of the mean speed of the Brownian motion of the nanobeads (500 nm) in the area with or without irradiation by two-photon excitation (740 nm).

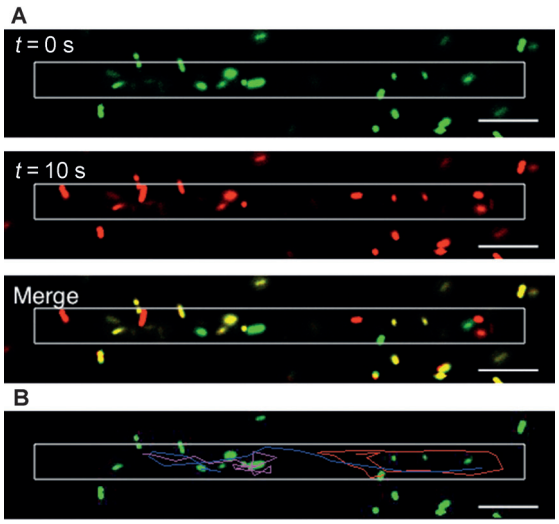

Figure 4. 3D spatial control of the chemotaxis of *E. coli* (RP437). A) Time-lapse CLSM images of the SYTO9-labeled *E. coli* in the DMACmoc-FF(CF_3) (0.075 wt%) gel. The inset rectangle was irradiated with focused laser light (740 nm). The fluorescence images were converted into pseudocolors (green: $t = 0$ s, red: $t = 10$ s) and merged. B) Three different tracks of each *E. coli* in the irradiation area shown in different colors. Scale bar: 10 μm (see also Movie S2 in the Supporting Information).

Movie S2 in the Supporting Information). The rate of the bacterial chemotaxis was evaluated to be $(6.6 \pm 1.0)\ \mu m\ s^{-1}$, which is comparable to the literature value.[18] In sharp contrast, one-photon irradiation of DMACmoc-FF(CF_3) with UV light (405 nm, 30 mW) did not restore the chemotaxis of the entrapped bacteria (see Figure S9 in the Supporting Information), because of its high toxicity, which highlighted the advantage of two-photon excitation by NIR light for manipulating living materials.

In summary, we successfully developed the first two-photon-responsive supramolecular hydrogel which can be readily fabricated with high spatial resolution by excitation with two-photon NIR light. As a consequence of the high biocompatibility of the two-photon NIR fabrication and the sufficient stiffness of the nanomesh composed of supramolecular fibers, the bacterial chemotaxis was remotely regulated in an off–on mode. The current supramolecular material is potentially useful for remote manipulation of not only the chemotaxis of bacterial cells but also the action of mammalian cells, such as migration and differentiation or controlled drug release in deep tissue. Further research towards these goals is currently in progress.

Received: April 10, 2014
Published online: May 27, 2014

Keywords: gels · photochemistry · self-assembly · supramolecular chemistry

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