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Thermally Responsive Supramolecular Nanomeshes for On/Off Switching of the Rotary Motion of F_1-ATPase at the Single-Molecule Level

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Abstract: The artificial regulation of protein functions is essential for the realization of protein-based soft devices, because of their unique functions conducted within a nano-sized molecular space. We report that self-assembled nanomeshes comprising heat-responsive supramolecular hydrogel fibers can control the rotary motion of an enzyme-based biomotor (F_1-ATPase) in an on/off manner at the single-molecule level. Direct observation of the interaction of the supramolecular fibers with a microbead unit tethered to the F_1-ATPase and the clear threshold in the size of the bead required to stop ATPase rotation indicates that the bead was physically blocked so as to stop the rotary motion of ATPase. The temperature-induced formation and collapse of the supramolecular nanomesh can produce or destroy, respectively, the physical obstacle for ATPase so as to control the ATPase motion in an off/on manner. Furthermore, this switching of the F_1-ATPase motion could be spatially restricted by using a microheating device. The integration of biomolecules and hard materials, interfaced with intelligent soft materials such as supramolecular hydrogels, is promising for the development of novel semi-synthetic nano-biodevices.

Keywords: ATPase · gels · nanostructures · single-molecule studies · supramolecular chemistry

Introduction

Progress in molecular biology and chemical synthesis has enabled the construction of many bio- and artificial molecules with nanometer precision. Proteins are unique in their various sophisticated functions as biocatalysts, biocarriers/-transporters, recognition/sensors, and energy/signal transducers, all carried out within nano-sized spaces. Studies of hybrid systems that combine biomolecules with artificial substances are a promising approach for innovative soft nanodevices.[1] As a pioneering example, Montemagno reported that the integration of a biomotor with nanofabricated inorganic substrates produced a unique nanodevice.[2] However, the simple combination of inorganic hard materials with soft biomolecules often diminishes the native performance, because the system has not evolved optimally. Rational strategies to optimize hybridized functions are now desired.

We recently developed a supramolecular hydrogel that can entrap proteins and enzymes without denaturation by providing semi-wet conditions.[3,4] In addition to such a passive role, the supramolecular materials might play an active role in the manipulation of protein functions in sophisticated nano-bio-architectures.[5] This is because self-assembled materials composed of small molecules generally show drastic and various changes in morphology from nano- to micrometer scale in response to external stimuli.[4] We herein report that a supramolecular soft material can act as both an effective matrix to regulate an enzyme motion in an on/off manner in response to external temperature, and as a hold-

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ing matrix. A thermally responsive supramolecular hydrogel consisting of entangled supramolecular gel fibers can regulate the rotary motion of F_1-ATPase, a motor protein,[6] in an on/off manner. By direct observation of a single molecule, we demonstrate that a microbead tethered to F_1-ATPase was trapped by the fiber network to effect the stop of the rotation. The formation and collapse of the network (so-called mesh) structure in response to external temperature can reversibly regulate the rotary motion of F_1-ATPase at the single-molecule level. This on/off switching of the F_1-ATPase rotation was carried out not only in bulk, but also within a limited space.

Results and Discussion

On/off switching of the F_1-ATPase rotation coupled with thermally responsive formation and collapse of the supramolecular nanomeshes: A thermally responsive supramolecular hydrogel consisting of a glycolipid-based hydrogelator **1** (Figure 1a) was employed as an intelligent semi-wet matrix for covering the F_1-ATPase. As previously reported,[3b] the macroscopic gel-to-sol phase transition of the hydrogel comprising **1** occurs by elevating the temperature (see Supporting Information Figure S1a). Below the gel–sol transition temperature (T_g, 48 °C), TEM (transmission electron microscopy) clearly showed that many gel fibers of width less than 10 nm entangled to form into mesh structures (Figure 1b). The nano-sized meshes of supramolecular hydrogel **1** are destroyed above T_g, which is in sharp contrast to the conventional polymeric gel fibers that are permanently stable because of their covalent linkage. This gel–sol transition is reversibly repeated many times (see Supporting Information Figure S1b). Owing to such formation and collapse in response to external temperature, the present nanomeshes may potentially become a unique matrix for controlling enzyme motion.

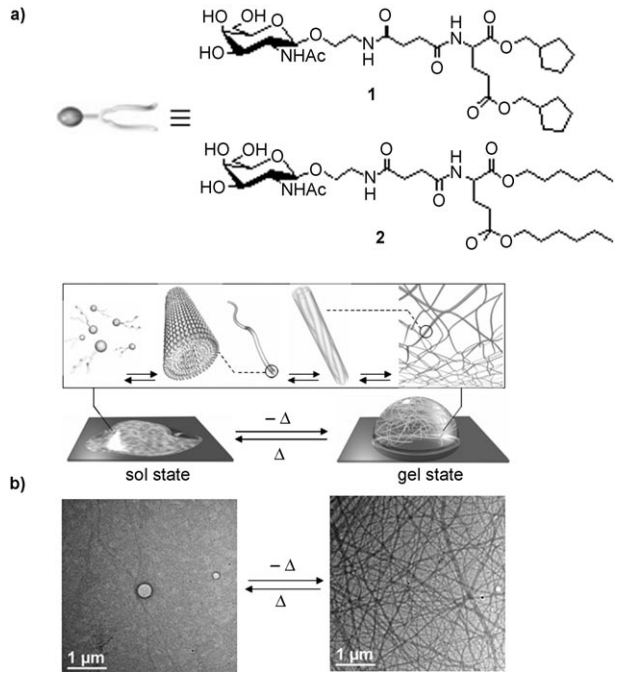

Figure 1. Thermally responsive gel–sol transition of the supramolecular hydrogel. a) Schematic illustration of the sol–gel transition and the molecular structure of supramolecular hydrogelators (**1**, **2**) used in this study. b) TEM images of the hydrogel **1** at RT (right) and the corresponding sol state heated above 60 °C (left). TEM observation was carried out without staining ([gelator **1**] = 0.35 wt % in 50 mM MOPS-KOH buffer (pH 7.1) containing 50 mM KCl).

ATPase, a rotary motor protein, was employed as a model protein because the observation method of the characteristic rotary motion at the single-molecule level has been established and the motion is tightly coupled to its enzymatic activity. According to previous reports,[7] F_1-ATPase was immobilized on the bottom of a glass plate through the $\alpha_3\beta_3$ ring in a flow chamber. A microbead was then attached to the γ subunit, a rotor axis of F_1-ATPase, through a biotin/avidin connection in order to visualize the F_1-ATPase rotation at the single-molecule level. The rotation behavior of the microbead tethered to F_1-ATPase during the gel-formation process was observed in a bright field by optical microscopy (see Supporting Information Figure S2), immediately after the buffer solution including the sol state of the hydrogelator **1** was infused into the flow chamber. After several tens of minutes, the rotation of a microbead larger than 450 nm in diameter stopped suddenly, as shown in Figure 2a and b. The stopped beads did not rotate again at 25 °C. However, when the hydrogel was heated above the T_g (48 ± 2 °C) for 2 min, the rotation of the F_1-ATPase immediately restarted (Figure 2b and Supporting Information Figure S3). The restarted rotation stopped again within several minutes after cooling to 25 °C, and it was restarted again by external heating at 60 °C (Figure 2b). This on/off switching of rotation was reproducibly observed at the single-molecule level in other F_1-ATPase molecules and in other batches. The repeated restart and pause of the F_1-ATPase rotation by heating and by cooling implies that the stopping process was neither due to the denaturation of the F_1-ATPase nor the detachment of the microbead from the γ subunit by the denaturation of the connecting avidin.

Furthermore, we found that the on/off regulation of ATPase is greatly dependent on the size of the beads attached to the ATPase. As shown in Figure 2c, among 52 ATPase molecules, all those with beads larger than 450 nm stopped rotating within 30 min, whereas 62 % of the ATPase molecules with beads smaller than 450 nm continued to rotate freely. This suggests that the empty voids of the nanomesh are around 450 nm. If the rotational space of the beads is larger than the void, the nanomesh may become a physical obstacle to ATPase rotation. However, ATPase does not experience such obstruction when the rotational space of the beads is smaller than the void. Additionally, we did not find a significant reduction in the rotational velocity after gelation in the case of the smaller beads attached to F_1-ATPase (Figure 2d). This suggests that ATP, a substrate of ATPase, may be too small to be tightly fixed in the nano-

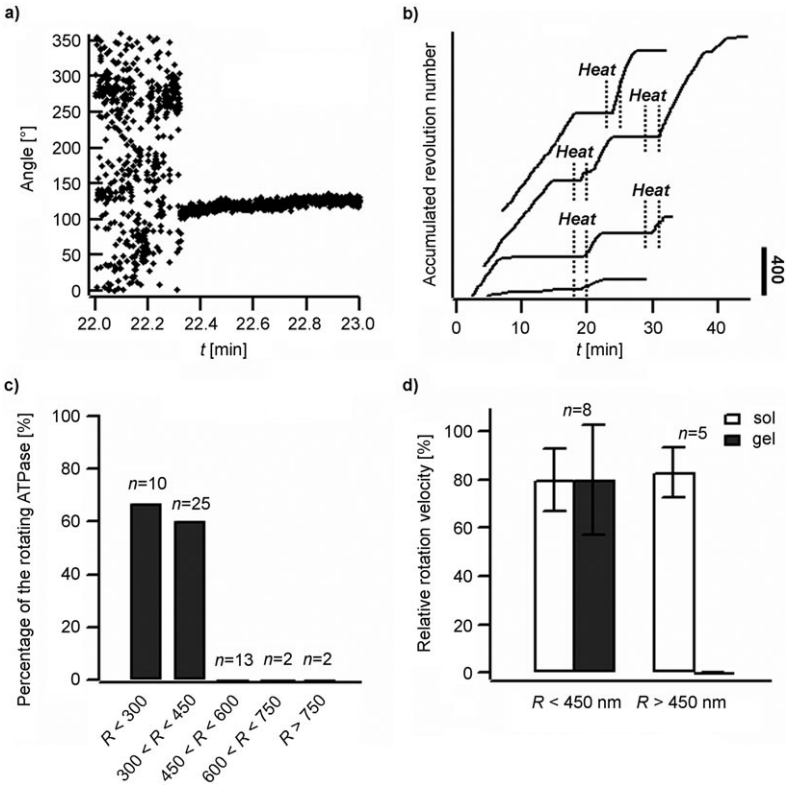

Figure 2. The rotary motion of a microbead attached to F_1-ATPase in the sol and gel states observed at the single-molecule level. a) Time trace of the rotation-angle change of microbead attached to F_1-ATPase before and after stopping. b) Time courses of the accumulated rotation number of F_1-ATPase under heat-switching experiments. The rotation was counted by an image analyzer system every 0.033 s. The rotation of F_1-ATPase stopped and paused for more than 5 min at 24±2°C, and then it restarted by the gel–sol transition that was induced by heating at 60°C for 2 min (between two dotted lines in the figure). c) Dependence of the ATPase rotation on the bead size (R) in the gel state. The percentage of the continuously rotating ATPase was determined by dividing the number of rotating beads in the gel state (30 min after the sol injection) by the total number of rotating beads in the sol state. d) The relative rotation velocity for the sol state (5 min after sol injection) and for the gel state (30 min after sol injection). The value was calculated by dividing the velocity for sol or gel by that in the aqueous solution. The microbeads with a mean bead size of 220 and 670 nm were used ([gelator **1**] = 0.35–0.40 wt% in 50 mM MOPS-KOH buffer (pH 7.1) containing 50 mM KCl, 2 mM ATP, and 2 mM $MgCl_2$).

meshes, and the ATP flux change by the gelation, if any, was not a significant factor affecting rotation.

Direct observation of the blockage of the F_1-ATPase rotation by the supramolecular meshes: To investigate in detail how the rotary motion of the F_1-ATPase is blocked in the gel state we used hydrogelator **2** that forms gel fibers thick enough to be visible by microscopy, although the homogeneous gel meshes cannot be formed. After the infusion of the sol of gelator **2** to the flow chamber, the rotation of a single molecular F_1-ATPase was carefully observed in real time. After about 30 min the gel fibers emerged as a bleary image at the focal point of the objective lens, and the growing fibers were gradually attached to the bottom of the glass plate (Figure 3a). During the fiber formation, the F_1-ATPase (motor A) continuously revolved. After 48 min a rotating microbead tethered to the F_1-ATPase frequently collided with the clearly emerged gel fibers, and after a short pause of about two seconds it stopped completely (Figure 3b and Supporting Information Figure S4). Figure 3c shows the time course of the revolutions of three distinct F_1-ATPases (motors A, B, and C) in the same visualized area. F_1-ATPase molecules revolved at a roughly constant speed until just before the collision with a fiber, then suddenly stopped rotating after the collision. Another revolving F_1-ATPase (motor B) stopped in the same way by collision with a different fiber two minutes later than the first F_1-ATPase (motor A). In contrast, the third F_1-ATPase (motor C) continued to revolve without pause in a place in which no fibers made contact with it, due to the inhomogeneous and lower density (and consequently large empty void) of the meshes (see Supporting Information Figure S5). Such direct observation strongly indicated that the stopping of the F_1-ATPase rotation can be ascribed to the physical capture of the bead-appended F_1-ATPase in the entangled gel-fiber meshes (Figure 4). Unlike the gel fiber **2**, it is unfortunate that the hydrogel fibers **1** are too thin to be detected by microscope in the bright field. Thus, we cannot directly observe the interaction of the fibers **1** with ATPase at the single-molecule level in real time. However, the very sharp stopping behaviors of ATPase observed for both gels **1** and **2** is consistent with the physical blockage of ATPase rotation. The clear size dependence of the beads for stopping ATPase rotation observed in the case of gel **1** is also in good agreement with the idea that the nanomeshes are physical obstacles for the rotation of the beads-tethered ATPase.

Regulation of the F_1-ATPase rotation in a restricted small area by using a microheating device: Interestingly, it is also possible to conduct the on/off switching of the F_1-ATPase motor in a restricted small area by combining the thermoresponsive hydrogel with a microheating device. We recently fabricated a microdevice consisting of a Ni electrode by using the microelectromechanical (MEMS) technique, which can heat a restricted small area around the Ni-pattern (Figure 5a).[8] In a flow chamber on this microdevice we immobilized F_1-ATPase attached to a microbead and this was

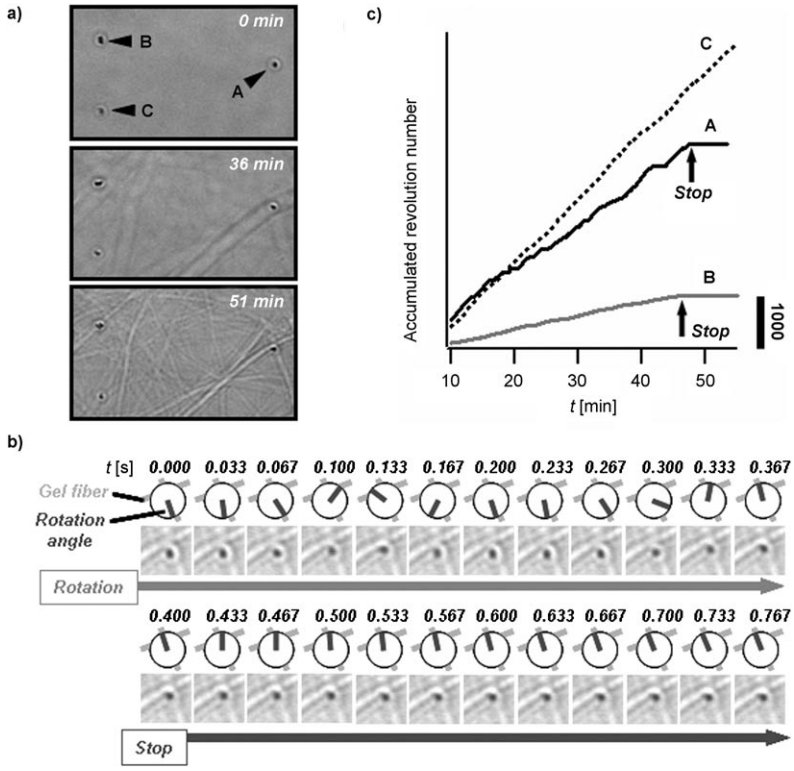

Figure 3. The mechanical blockage of the revolution of a microbead attached to F_1-ATPase by the hydrogel fibers. a) The bright-field images of the microbead and the emerging gel fibers obtained by optical microscopy at RT. Black arrows point to three F_1-ATPase molecules (motors A, B, and C) tethering a microbead. The time since the injection of the sol state of the hydrogelator **2** is shown. b) The sequential enlarged images of the rotating and stopping microbead A just before and after the collision. Above each image the illustration for the rotation angle of the F_1-ATPase is added for clarity. c) Time courses of the accumulated revolution numbers of the F_1-ATPase motors A, B, and C were plotted every 0.033 s ([gelator **2**] = 0.60 wt% in 50 mM MOPS-KOH buffer (pH 7.1) containing 50 mM KCl, 2 mM ATP, and 2 mM $MgCl_2$).

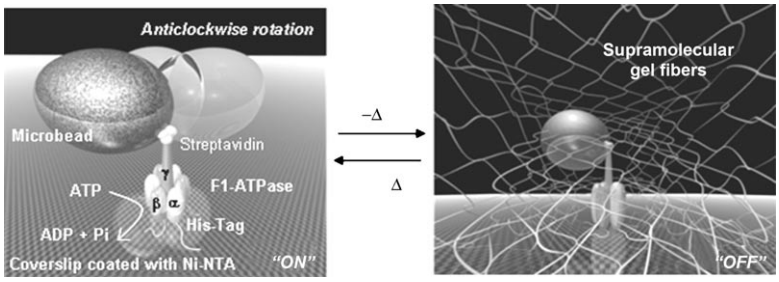

Figure 4. Schematic illustrations of the on/off switching of F_1-ATPase rotation by entanglement of the stimuli-responsive supramolecular hydrogel fibers (nanomeshes). To clearly show the component of rotary motor, F1-ATPase is represented enlarged.

then covered with the hydrogel **1**. Upon gel formation, all F_1-ATPases completely stopped rotation over the entire area of the device surface, independent of their position. By heating the microdevice, the rotation immediately restarted at the surface just around the Ni electrode (Figure 5b), whereas the other F_1-ATPase molecules located far from the Ni-electrode remained motionless (see Supporting Information Figure S6). Thus, hybridizing the supramolecular hydrogel by using the MEMS technique realized the spatially selective on/off control. Notably, the response time was significantly improved in this MEMS-device system relative to the bulk heating system, due to the effective heat transmittance in the microspace.

Conclusion

We demonstrated that the physical capture and release of the microbeads-appended enzyme by the stimuli-responsive nanomeshes is promising for the perfect on/off switching of enzyme rotation. In this system, it is conceivable that the microbead tethered to the F_1-ATPase motor acts as a knob that can transduce the physical restriction/relaxation given by the nanomeshes to the enzyme's active center. On the other hand, the supramolecular nanomeshes operate as an intelligent hand of nano/micro size that can appear or disappear in order to grip or release, respectively, the knob in response to the external temperature. There are several reports of switching the enzyme motion based on the direct incorporation of a switching unit such as a metal binding site, magnetic bead or photochromic artificial molecules into the protein framework through a covalent bond, for response to a chemical stimulus, magnetic field or visible light.[9–11] Recently, Ionov et al. successfully switched kinesin motion on a thermoresponsive polymer-grafted surface by hindering the binding of microtubules with the thermoinduced extension of polymer chains.[12] The present strategy is unique in that soft materials entrapping the enzyme can be used as an active operator. The supramolecular nanomeshes can selectively regulate the movement of microscale objects without disturbing the diffusion and activity of nanoscale biomolecules, and thereby are promising matrices for versatile application in the manipulation of micro-biomachines powered by biological motors such as kinesin- or myosin-based molecular shuttles and living bacteria.[11a,12] The elaborate combination of engineered enzymes

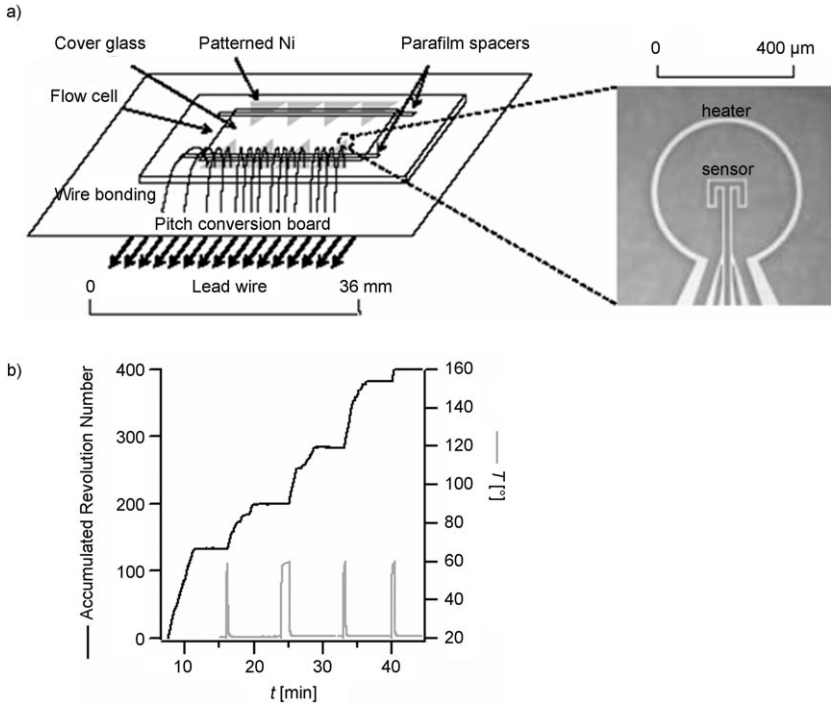

Figure 5. a) Macroscopic (left) and microscopic (right) views of the integrated Ni-patterned microheater and microsensor on a glass plate used in this study. b) The control of the revolution of F_1-ATPase using a Ni-patterned microheater. The accumulated revolution number of the F_1-ATPase (black solid line) and the local temperature (gray solid line) near the microheater is plotted against the time.

with nanofabricated hard substrates through intelligent soft materials such as the supramolecular nanomeshes should facilitate the development of novel protein-based molecular devices.

Experimental Section

General: All gelators were prepared according to our synthetic method reported previously.[3b] Reagents for the synthesis of gelators were obtained from Kishida Chemical, Watanabe Chemical Industries, Wako, or TCI (Japan). A mutant $\alpha_3\beta_3\gamma$ subcomplex (α-C193S, β-His-10 at N-terminus, γ-S107C/I210C) from a thermophilic *Bacillus* PS3 (referred to as F_1-ATPase) was expressed and purified as described elsewhere.[9a] Streptavidin-coated magnetic beads (Seradyn; normally 0.73 μm) were sonicated to disperse in a suspension and lightly centrifuged as described.[7a] A microheater and a microthermoresistive sensor integrated on a glass plate were prepared by patterning nickel on the glass plate (Matsunami; 24×32 mm) as previously reported.[8a,b]

Rotation assay: A flow chamber for the rotation assay was constructed from two uncoated glass plates (Matsunami; a top cover glass was 18 mm×18 mm, and a bottom one was 24 mm×32 mm) sandwiching two parallel strips of greasy paper as spacers.[7b] In the rotation assay experiments, a bottom glass coated with Ni^{2+}-NTA (NTA = nitrilotriacetate) was used to strengthen the immobilization of F_1-ATPase.[7a] F_1-ATPase molecules were immobilized on the glass plates in a flow chamber and subsequently modified with magnetic beads according to the method reported previously.[7b] All rotating assays in the sol or gel state were started by infusion of a heat-dispersed gelator in buffer A (50 mM 3-(*N*-morpholino)propanesulfonic acid-KOH, pH 7.1/50 mM KCl) supplemented with Mg-ATP (2 mM), and the rotating beads were observed as bright images at the single-molecule level. The images were videotaped every 0.033 s and analyzed by using a custom software.[7b]

Heating by a plate heater: A flat plate heater (Tokai Hit MATS-75R; 75 mm in radius) was set above the flow chamber containing F_1-ATPase and a heat-responsive gelator **1** solution (0.35 wt%), and a water drop was injected into the space between the plate heater and the flow chamber to conduct heat effectively from the heater to the gelator solution. The gel state was melted by heating (temp. was raised at approximately 30 °C/min (from 24 to 60 °C for 2 min (see Supporting Information Figure S3)).

Heating by a microheater: Two parallel strips of greasy paper were placed on the Ni-patterned glass plate, and an uncoated cover glass was put on the strips to form a flow chamber. In this flow chamber, a rotation assay of F_1-ATPase in the gelator **1** solution (0.50 wt%) was performed as described above. The gel just above the microheater was locally heated by the Joule heat caused by the electric current in the Ni pattern. The temperature of the heated space was obtained by measuring the resistance of the Ni pattern for the microthermosensor as reported previously.[8] To induce the gel–sol transition, the heating temperature was set at higher than 55 °C (above T_g).

Observation of gel structure by microscopy: After the stable hydrogel formation, the carbon-coated copper grid was dipped in this gel, and this grid was immediately dried in vacuo for 12 h at RT. To obtain the image of the gelator solution in the sol state, the carbon-coated copper grid was dipped in the heat-dispersed gelator solution and was rapidly dried in vacuo. The TEM observation of the prepared sample was carried out without staining under accelerating voltage of 120 kV. TEM images were recorded by using a JEOL-JEM-2010 apparatus.

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