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Hydrophilic Fluorescent Nanogel Thermometer for Intracellular Thermometry

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The temperature of a living cell is changeable during every cellular event, such as cell division, gene expression, enzyme reaction, and metabolism. In 1932, for instance, significant thermogenesis was found in ATP hydrolysis,[1] underlying the present bioenergetics. In mitochondria, surplus energy in ATP production is released in the form of heat, and a consequent rise in temperature coordinates other cellular events.[2] From a clinical viewpoint, pathological cells are warmer than normal cells because of their enhanced metabolic activity.[3] Thus, measuring cellular temperature can contribute to the explanation of intricate biological processes and the development of novel diagnoses. Here we demonstrate for the first time intracellular thermometry with a newly developed fluorescent nanogel thermometer **1**, which is superior to other candidate thermometers[4−8] in terms of biocompatibility (i.e., size,[4,5] sensitivity,[6] and solubility[7]) and negligible interactions with cellular components.[8] Intracellular temperature variations associated with biological processes can be monitored with a temperature resolution of better than 0.5 °C using **1**.

Figure 1A shows a schematic diagram of the fluorescent nanogel thermometer **1**. The fluorescence switching of **1** is based on a strategy we previously established:[7a] the thermoresponsive polyNIPAM unit[9] was combined with a water-sensitive fluorophore (i.e., DBD-AA units[10] in this case). In addition, two arrangements were made to **1** in view of its need to function in intracellular environments. The first was gelation at the nanometer scale by an emulsion polymerization technique using a cross-linker (MBAM).[7b] The inclusion of fluorescent units in a nanogel can remove undesirable fluorescence enhancement/quenching due to chemical interactions between cellular components and the fluorophores. The second was the unique enrichment of ionic sulfate groups by using an extraordinary quantity of initiator ammonium persulfate in the preparation of **1** (i.e., the ammonium persulfate/NIPAM molar ratio was 0.28, while that in common procedures is 0.01−0.046).[7b,11] It is striking that conventional nanogels[7b] could not be applied in intracellular thermometry because of precipitation in the culture medium. In the present study, the highly hydrophilic surface created by the sulfate groups protected **1** from precipitation at high ionic strength and from localization on the cytoplasmic membrane.

The performance of **1**[12,13] in electrolytes was assessed using a spectrofluorometer. Figure 1B indicates the fluorescence response of **1** to the change in medium temperature at different ionic strengths. Potassium chloride was used as the ion source to mimic intracellular conditions, as K^+ is the most abundant ion in the cytoplasm (∼139 mM).[14] As shown in Figure 1B, fluorescence enhancement of **1** with increasing temperature was observed in all measurements. As the KCl concentration increased from 0 to 100 mM, the response curve became sharper, producing a larger

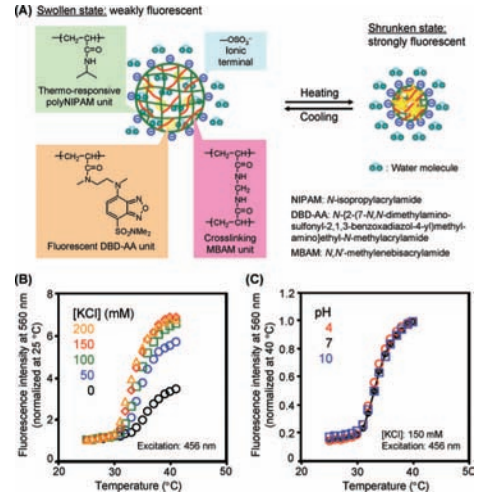

Figure 1. Fluorescent nanogel thermometer **1**. (A) Schematic diagram and chemical structures of the components. At a lower temperature, **1** swells by absorbing water into its interior, where the water-sensitive DBD-AA units are quenched by the neighboring water molecules. When heated, **1** shrinks with the release of water molecules, resulting in fluorescence from the DBD-AA units. (B) Fluorescence responses to temperature variation in water and in KCl solutions. (C) Insensitivity to pH variation. HCl and KOH were used for the pH adjustments. The fluorescence quantum yield was 0.37 at 40 °C.

fluorescence enhancement upon heating. In contrast, an almost identical response to the temperature variation was obtained when the KCl concentration was in the range 100−200 mM. This feature is desirable because the total ion concentration in the cytoplasm is equivalent to 150 mM.[14] The fluorescence response of **1** was independent of environmental pH, as indicated in Figure 1C. This is also advantageous because local pH in living cells is affected by neighboring structures and can easily vary by as much as one unit.[15] Furthermore, the performance of **1** was not even affected by a surrounding protein (e.g., bovine serum albumin; see Figure S4 in the Supporting Information). All of these results imply that **1** has a high capacity for accurate intracellular thermometry.

In this study on intracellular thermometry, COS7 cells were adopted as the subject of **1**. Highly hydrophilic **1** could easily be introduced into the cytoplasm by a microinjection technique without precipitation. Figure 2A shows representative phase-contrast and fluorescence images of living COS7 cells containing **1**. During the experiment, photobleaching and leaking of **1** were not problematic.[16] As shown in Figure 2A, **1** emitted fluorescence as dots at higher temperatures. These images show that **1** was dispersed within the cytoplasm but did not move to the nucleus. Figure 2B (left axis) indicates the calibration curve for intracellular thermometry with **1** (the relationship between total fluorescence intensity of **1** within a single COS7 cell and temperature). The total fluorescence intensity was obtained from fluorescence images by summing the fluorescence intensities of all the pixels within a single

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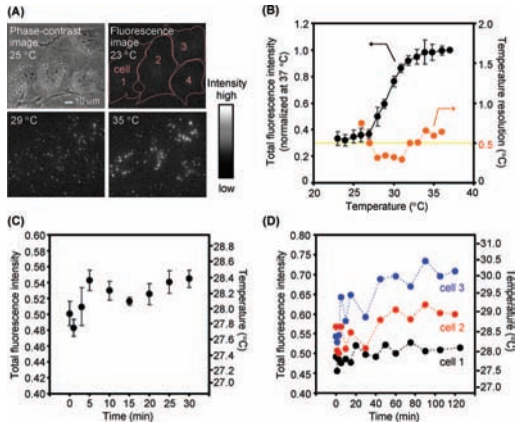

Figure 2. Intracellular thermometry with the fluorescent nanogel thermometer **1**. (A) Phase-contrast and fluorescence images of living COS7 cells containing **1**. Indicated temperatures were those of the culture medium as measured by a thermocouple. (B) Calibration curve (black, left axis) and temperature resolution (orange, right axis). The total fluorescence intensity of a single cell ($n = 4$, mean ± s.d.) was adopted as a temperature-dependent parameter by **1**. (C, D) Changes in the total fluorescence signal of **1** in a living COS7 cell (left axis) and the corresponding temperature (right axis) after the additions of (C) FCCP (finally 100 μM, $n = 3$, mean ± s.d.) and (D) camptothecin (finally 2 μM) to the culture medium at $t =$ 0 min. All of the experiments were performed with excitation at 488 nm and detection at 515−550 nm. In (C) and (D), the total fluorescence intensity of each cell was measured at 37.0 °C beforehand for normalization, and the temperature of the culture medium was maintained at 28.0 °C during the experiment.

COS7 cell. As Figure 2B shows, the fluorescence signal of **1** sharply changed between 27 and 33 °C in the COS7 cells.[17,18] Next, the temperature resolution (i.e., the minimum temperature difference to be significantly discriminated) was evaluated for **1** in the COS7 cells. As a result, the temperature resolution in thermometry with **1** was 0.29−0.50 °C over the range 27−33 °C (Figure 2B, right axis). Negative control experiments[16] using a temperature-insensitive poly(*N*-ethylacrylamide)[19]-based nanogel in COS7 cells denied the possibility that the fluorescence enhancement of **1** with increasing temperature was due to other cellular environmental changes as a consequence of the heating.

For a more practical use of **1**, intracellular temperature variations induced by external chemical stimuli were considered. The first case used the carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP).[20] In the literature, FCCP is an uncoupler of oxidative phosphorylation in mitochondria, causing heat production by respiration.[2a] Figure 2C indicates the time course of the fluorescence signals of **1** in COS7 cells exposed to FCCP (left axis) and the corresponding temperatures obtained using the calibration curve (right axis). No noticeable morphological changes were observed on the tested COS7 cells during the experiment.[16] It can be seen in Figure 2C that FCCP provoked the fluorescence enhancement of **1**, i.e., intracellular heating by 0.45 °C increments for 30 min. This temperature variation exceeded the temperature resolution of **1** (0.31 °C) at ∼28 °C (see Figure 2B), allowing us to affirm that our thermometer **1** successfully detected the intracellular temperature increase due to the FCCP-induced uncoupling. The case using camptothecin[21] is shown as an unveiled temperature variation, which might be due to early responses of apoptosis such as DNA cleavage. Figure 2D indicates the time courses of the fluorescence signals from **1** in three COS7 cells exposed to camptothecin and the corresponding intracellular temperatures (for morphological changes, see Figure S10 in the Supporting Information). Interestingly, the pattern of temperature variation was differed among the cells, which may be related to the cell-division-dependent effects of camptothecin.[21] Thus, energy balances in various kinds of cellular events would be defined by the novel fluorescent nanogel thermometer **1**.

In conclusion, intracellular thermometry is now practical using the novel fluorescent nanogel thermometer **1**. With increasing temperature, **1** in the cytoplasm produces stronger fluorescence, through which a temperature difference of less than 0.5 °C can be distinguished without any interference due to precipitation or interaction with cellular components. The functional temperature range[7b] and fluorescence color of the thermometer can be modified by replacing the gel component(s). Applications to fluorescence lifetime imaging[10] and organelle-specific thermometry with a localization signal are developments expected in the near future.

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Supporting Information Available: Experimental details and Figures S1−S10. This material is available free of charge via the Internet at http://pubs.acs.org.

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(11) This was confirmed by elemental analysis of sulfur. See the Supporting Information.
(12) A transmission electron microscopy image indicated that the average diameter of **1** was 51.2 ± 10.5 nm (mean ± s.d.) in the dried state. Dynamic light scattering and fluorescence measurements on **1** dispersed in water demonstrated a reversible heat-induced size variation and consequent fluorescence switching. See Figures S1−S3 in the Supporting Information.
(13) A related temperature-jump investigation revealed that heat-induced shrinking of a polyNIPAM nanogel occurs on a microsecond time scale. See: Wang, J.; Gan, D.; Lyon, L. A.; El-Sayed, M. A. *J. Am. Chem. Soc.* **2001**, *123*, 11284–11289.
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(16) For detailed data, see the Supporting Information.
(17) The response curve of **1** in the COS7 cells (2.6-fold fluorescence enhancement over the range 27−33 °C) was not identical to that in the 150 mM KCl solution (5.3-fold over the range 30−40 °C). This gap is possibly due to complexity of intracellular environments, and therefore, a calibration curve should be obtained for each cell species before intracellular thermometry.
(18) The estimated contribution of autofluorescence to the ordinate was <0.016.
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