

Intracellular Protein-responsive Supramolecules: Protein Sensing and In-cell Construction of Inhibitor Assay System

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ABSTRACT: Supramolecular nanomaterials responsive to specific intracellular proteins should be greatly promising for protein sensing and imaging, controlled drug release or dynamic regulation of cellular processes. However, valid design strategies to create useful probes are poorly developed, particularly for proteins inside living cells as targets. We recently reported a unique supramolecular strategy for specific protein detection using self-assembling fluorescent probes consisting of a protein ligand and a fluorophore on the live cell surface, as well as in test tube settings. Herein, we discovered that our self-assembled supramolecular probes having a rhodamine derivative (tetramethylrhodamine or rhodamine-green) can incorporate and stay as less-fluorescent aggregates inside the living cells, so as to sense the protein activity in a reversible manner. Using the overexpressed model protein (dihydrofolate reductase), we demonstrated that this turn-on/off mode is controlled by selective ligand-protein recognition inside the live cells. Not only such a model protein, but also endogenous human carbonic anhydrase and heat shock protein 90 were specifically visualized in living mammalian cells, by use of the similar ligand-tethered supramolecular probes. Furthermore, such reversibility allowed us to intracellularly construct a unique system to evaluate the inhibitors affinity toward specific endogenous proteins in live cells, highlighting the potential of dynamic supramolecules as novel intelligent biomaterials.

1 INTRODUCTION

Proteins are involved in all biological events, and as such, their localization, expression level and functions are dynamically regulated in live cells.^{1,2} Therefore, protein-responsive materials should hold promise for specific protein sensing, live imaging, controlled drug release, and regulation of cellular processes.^{3,4} The use of supramolecular nanomaterials in response to selective proteins in live cells is attractive owing to their dynamic reversibility and potentially high biodegradability.^{5,6} Cell surface proteins as therapeutic and/or diagnostic biomarkers are easily accessible for synthetic biomaterials and thus many promising nanomaterials have been developed.^{7,8} In contrast, intracellular proteins are known to be extremely difficult to target and the development of supramolecular nanomaterials that are responsive to “intracellular proteins” remains challenging. There are various obstacles that must be overcome for nanomaterials to be workable inside living cells, including sufficient cell permeability through biomembranes, high target protein specificity under crude intracellular conditions, and effective switching functions that are operable inside live cells. As pioneering works, enzymatic reaction-triggered self-assembling nanostructures that function inside living cells have been reported by Rao et al., in which an intracellular protease initiates the deprotection reaction of precursor molecules to yield covalently linked fluorescent oligomers.^{9,10} Xu et al. have succeeded in the formation of phosphatase-triggered supramolecular hydrogels in live cells, demonstrating their use

in an activity assay for the cytosolic enzyme.^{11,12} Aggregation-induced emission resulting in a turn-on fluorescence in response to a target enzyme was also recently applied to specific protein sensing in live cells.¹³⁻¹⁵ Although these examples indicate the successful formation of supramolecular nanostructures in living cells, reversible self-assembly/disassembly of such materials has never been achieved. Reversible self-assembly/disassembly would be a desirable feature of supramolecular nanomaterials, and could lead to various applications including dynamic protein sensing in response to the expression level, protein activity or protein-triggered intracellular drug release.

We recently developed a disassembly-driven turn-on nanoprobe, where the ligand-tethered fluorophores exhibited unique self-assembling features and showed a reversible turn-on fluorescent change toward non-enzymatic-proteins on a live cell surface, as well as toward purified ones in a test tube.¹⁶⁻¹⁹ However, we have not yet succeeded in demonstrating that our nanoprobe can penetrate the cell membrane efficiently, and bind selectively and reversibly to the target protein inside the cells. On the basis of elaborate molecular design and careful physicochemical analyses, we herein reveal that the self-assembling nanoaggregates can operate well inside living cells, that is, “intracellular” proteins detection and imaging were carried out with turn-on manner. From the model experiments using overexpressed dihydrofolate reductase (eDHFR), it was clear that (1) the probe can be incorporated into the inside cells by an endocytosis mechanism, and (2) self-assembly/disassembly of the

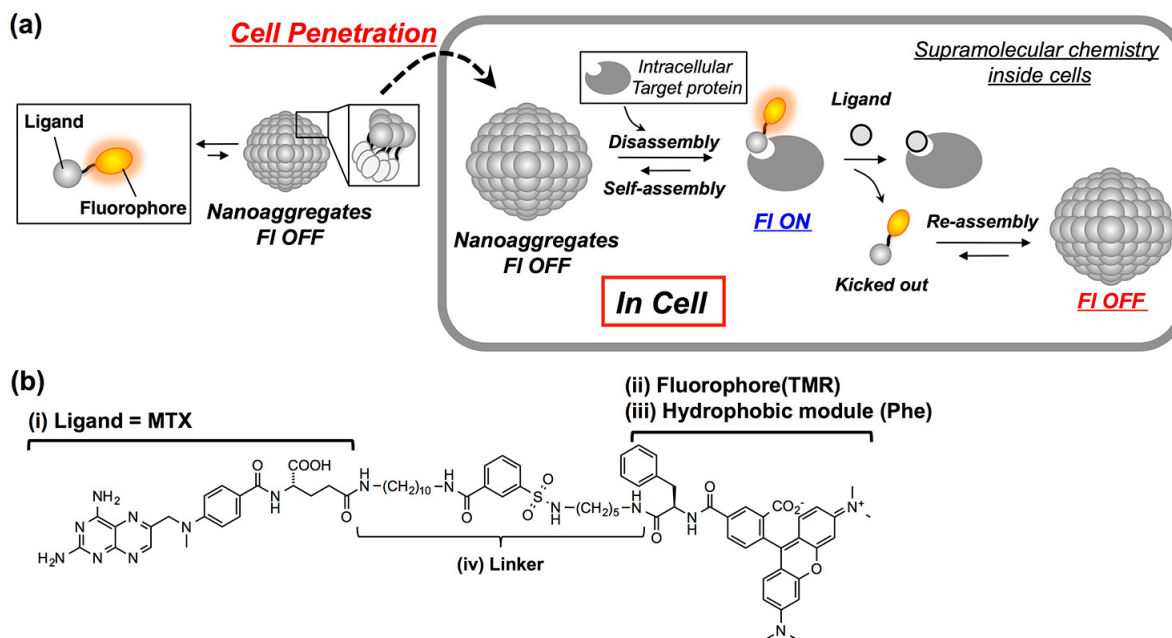


Figure 1. Protein-responsive nanoaggregates for specific and reversible intracellular protein sensing. (a) Schematic illustration of in cell reversible protein sensing by self-assembling nanoaggregates. (b) Chemical structure of ligand-tethered probe **1** for intracellular eDHFR imaging.

1 nanoaggregates indeed occurred reversibly in living cells upon
2 a specific ligand-protein recognition (Figure 1a). More
3 importantly, we also succeeded in specific imaging of
4 endogenously expressed intracellular proteins such as human
5 carbonic anhydrase (hCAII) and heat shock protein 90
6 (HSP90). An imaging-based inhibitor assay for these
7 endogenous proteins using live cells was constructed, clearly
8 highlighting the advantage of incorporating a reversible
9 assembly/disassembly function in the design of sophisticated
10 supramolecular biomaterials.

11 RESULTS & DISCUSSION

12 Self-assembling rhodamine-based supramolecule is 13 capable of turn-on sensing of eDHFR inside the liv- 14 ing cells.

15 We previously reported that our ligand-tethered probes bear-
16 ing self-assembling property allowed for rapid and convenient
17 turn-on fluorescent imaging of membrane-bound endogenous
18 protein on the live cell surface for a short-time observation. In
19 case of probe **1** having methotrexate (MTX) as a ligand for
20 membrane-bound folate receptor, for example, strong fluores-
21 cence could be detected only from the KB cell surface (endog-
22 enously expressing folate receptor) within 1 h. On the other
23 hand, we noticed that fluorescent granules were also weakly
24 observed inside of the cells for the longer incubation time
25 (about 12 h) for both KB cell and other cell line not-
26 expressing folate receptor. This indicated that the probes can
27 be internalized into live cells with retaining the suppressed
28 fluorescence, which suggested us an interesting possibility that
29 some of our self-assembling probes may be applicable even to
30 “intracellular protein” sensing. We thus initially examined the
31 self-assembly/disassembly behavior of the fluorescent probe **1**
32 inside of the living cells. In detail, this probe **1** is composed of
33 the following four modules: (i) methotrexate (MTX) as a hy-
34 drophilic ligand for targeting not only folate receptor, but also
35 eDHFR used here as an intracellular model protein, (ii) tetra-

36 methylrhodamine (TMR) as a zwitterionic fluorophore for
37 imaging, (iii) phenylalanine as a hydrophobic module for fine-
38 ly tuning the aggregation property, and (iv) a relatively hydro-
39 phobic linker for connecting these modules (Figure 1b).¹⁹ We
40 confirmed that probe **1** formed spherical or oval aggregates
41 with a mean diameter of 40 nm, and had a critical aggregation
42 concentration (CAC) lower than 1.31 μ M (Figure 2a, b and
43 Figure S1a, b). The fluorescence of probe **1** was greatly sup-
44 pressed owing to self-aggregation and was enhanced by 37-
45 fold upon addition of purified eDHFR in a test tube (Figure
46 S1c-e).¹⁹ The detection limit of **1** for eDHFR was 66 nM (Fig-
47 ure S1f).

48 A HeLa cell line stably over-expressing GFP-fused eDHFR
49 (HeLa-DG cell) was employed in this model study.²⁰ From the
50 confocal laser scanning microscopy (CLSM) image using the
51 GFP channel, it was evident that eDHFR-GFP was distributed
52 throughout the entire cell including the cytosol and nuclei.²⁰
53 When probe **1** was added to HeLa-DG cells and incubated for
54 12 h at 37°C, strong TMR fluorescence was observed by
55 CLSM from the intracellular regions without any washing,
56 which completely merged with the image obtained from the
57 GFP emission (Figure 2c). Under these conditions, serious
58 cytotoxicity was not observed (Figure S1g). The fluorescence
59 intensity inside the cells dramatically decreased upon addition
60 of trimethoprim (TMP), a strong inhibitor for eDHFR,^{21,22}
61 without any intensity changes in the fluorescence of the extra-
62 cellular region (Figure 2d). In a negative control using normal
63 HeLa cells lacking eDHFR-GFP, only fluorescent granules
64 were weakly observed (Figure S2a). On the basis of these re-
65 sults, it was concluded that **1** is capable of fluorescently imag-
66 ing eDHFR inside the HeLa cells in the turn-on mode through
67 MTX-eDHFR specific interactions. Quantitative evaluation of
68 the amount of **1** transported into the live cells revealed that
69 almost the same amount of probe **1** entered these cells regard-
70 less of the presence or absence of the target eDHFR, and the
71 incorporated probe **1** remained in the HeLa cell interior and
72 was not released, even in the presence of the inhibitor (TMP)

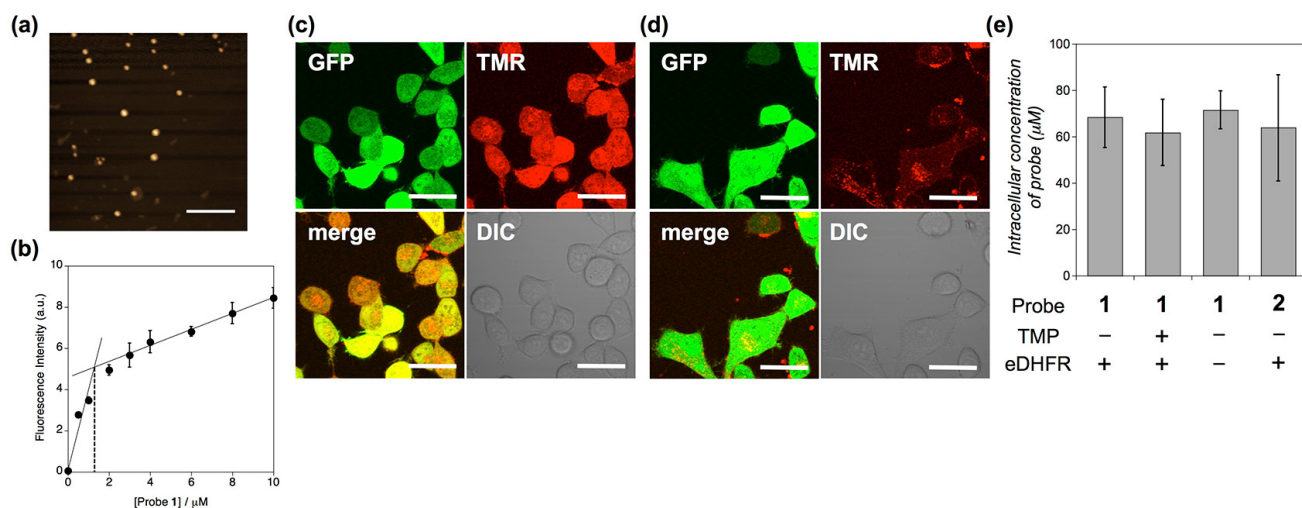


Figure 2. Fluorescent imaging of intracellular eDHFR-GFP using probe **1** in living cells. (a) Atomic force microscopy (AFM) image of the self-assembled probe **1** (10 μ M) (scale bar 500 nm). (b) Concentration-dependent fluorescent intensity change of **1** (0–10 μ M, λ_{em} = 584 nm). (c) CLSM images of HeLa-DG cells treated with probe **1** (2 μ M) for 12 h at 37°C. The images were obtained without any washing (scale bars, 20 μ m). (d) CLSM images of HeLa-DG cells treated with probe **1** (2 μ M) for 12 h at 37°C, and then treated with TMP (10 μ M). The images were obtained without any washing (scale bars, 20 μ m). (e) Intracellular concentration of incorporated probe **1** or **2** in HeLa-DG cells or normal HeLa cells, calculated with fluorescent spectra of these cell lysates. The experiments were performed in triplicate to obtain mean and standard deviation values (shown as error bars).

1 (Figure 2e). We also determined the concentration of **1** per single cell to be over 60 μ M, which was higher than the CAC of **1** (< 1.31 μ M, Figure 2b and e). Given all of the results here obtained, we considered that (1) the probe **1** was transported into the cells independent of eDHFR and self-assembled in the absence of eDHFR inside cells, (2) disassembly of the nanoaggregates was driven by ligand-eDHFR recognition in the presence of eDHFR, triggering the turn-on fluorescence change, and (3) **1** was released out of the eDHFR binding pocket by exchange with the competitive inhibitor, which resulted in the re-assembly of **1** inside the living cells. Consequently, the fluorescence was turned off although the weak granulated fluorescence was still present as background noise (Figure 1a).

In order to further evaluate any impacts of self-assembling property of the probe **1** on the cell-penetration efficiency and retention ability inside the cells, we employed an always-on (non-assembling) probe **1'** (Figure S3) and a self-assembled but non-recognizable probe **2** (under-mentioned). In case of the always-on-type probe **1'**, intracellular eDHFR cannot be clearly visualized mainly due to its high background signal from the extracellular region (Figure S3e). Also, the amount of incorporated probe **1'** per single HeLa-DG cell was too small to be determined by the method applied for the probe **1**, which was lower than 2 μ M.²³ On the other hand, in the case of probe **2** that is a non-recognition self-assembling probe tethering not eDHFR ligand but hCA ligand, almost the same amount of probe was incorporated in HeLa-DG cell (64 μ M, Figure 2e) as the case of probe **1**, regardless of no ligand/protein recognition. Given these results, it was clear that (i) the self-assembly property may contribute to both the enhanced cell-penetration and the probe retention inside the cells, and (ii) the protein binding does not strongly impact on the probe retention in cells. These should give important clues for designing supramolecular self-assembly workable in live cell systems.

36 Mechanisms of internalization and turn-on sensing of probe **1** with eDHFR in cells.

To explore the transport mechanism of the self-assembling probe **1** into the HeLa-DG cells, time-lapse fluorescent live imaging was performed. As shown in Figure 3a, upon addition of probe **1** (after a few minutes), weak fluorescence was only observed from the cell membrane, and after 20 min at 37°C, several fluorescent granules subsequently appeared inside the cells. The punctum fluorescence gradually became more homogeneous and was distributed in the whole cytosol. The cytosolic fluorescence gradually increased in intensity until 12 h, while the membrane fluorescence relatively lessened and was indistinguishable from the cytosolic one (Figure 3b). In the case of cells treated with **1** at 4°C, the intracellular fluorescence was negligible even after a 5 h incubation, whereas weak fluorescence was observed on the cell membrane (Figure S2b). On the other hand, weak but distinct fluorescent granules were observed from normal HeLa cells (lacking eDHFR-GFP) as shown above merged with that of LysoTracker green (Figure S2c). These results strongly suggested that endocytosis is the main pathway for the intracellular transport of probe **1**. More detailed analysis was conducted using various endocytosis inhibitors. After treatment of genistein or 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA), inhibitors for caveolae-mediated endocytosis²⁴ or macropinocytosis, respectively,^{25,26} the fluorescence intensities of the cells stained with **1** were suppressed by less than the half compared with the cells incubated in the absence of inhibitors (Figure S4). In contrast, a decrease in fluorescence was not observed upon incubation with amantadine, an inhibitor for clathrin-mediated endocytosis.²⁷ Taken together, it suggested that **1** (or self-assembling nanoaggregates of **1**) internalized through caveolae-mediated endocytosis or macropinocytosis, both of which had been recently characterized as primary pathways for internalization of macromolecules such as proteins or metal nanoparticles.^{28,29} Thereafter, the internalized **1** would gradually escape from the endosomal vesicles to the cytosol. In the absence of the target eDHFR, most of **1** formed stable non-fluorescent aggregates whereas the aggregates disassembled into fluorescent **1**/eDHFR complexes in the presence of eDHFR, resulting in recognition-driven turn-on fluorescence inside the cells.

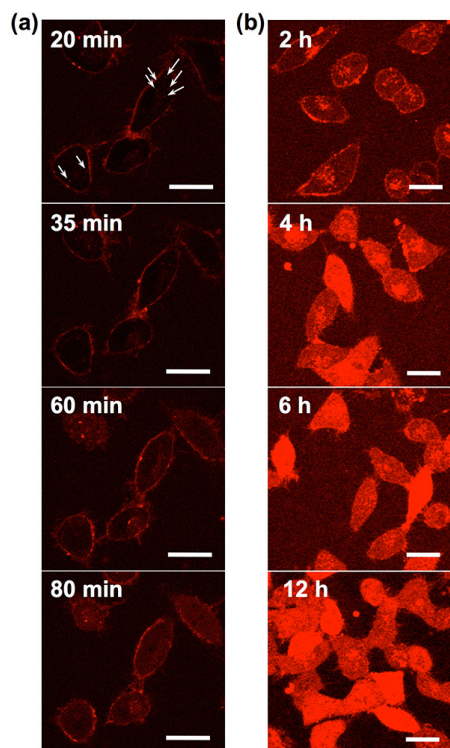


Figure 3. Time trace of eDHFR imaging in HeLa-DG cells stained by probe **1** incubated at 37°C within (a) a short time period (20 to 80 min) and (b) a long time period (2 to 12 h). Scale bars, 20 μ m.

1 Intracellular endogenous hCAII sensing and in-cell 2 inhibitor assay by self-assembling supramolecular 3 probe.

4 The present success in imaging intracellular eDHFR as a
5 model encouraged us to target more difficult proteins, that is,
6 “endogenous” proteins inside of living cells. Cytosolic hCAII,
7 a protein related to various diseases such as epilepsy or glau-
8 coma was attempted. Using the same design strategy as the
9 eDHFR probe, a TMR-tethered probe **2** carrying a benzenesul-
10 fonamide ligand, a selective inhibitor of the hCA family,³⁰ was
11 prepared (Figure 4a). Atomic force microscopy (AFM) or
12 dynamic light scattering (DLS) data revealed the formation of
13 spherical or oval aggregates of **2** with a mean diameter of 100
14 nm in aqueous buffer (Figure 4b and S5a). The CAC value of
15 **2** was determined to be lower than 110 nM (Figure S5b). We
16 also confirmed that probe **2** showed a 30-fold increase in the
17 fluorescent intensity in response to purified hCAII in a test
18 tube (Figure 4c and S5c, d). The detection limit of **2** for hCAII
19 was 67 nM (Figure S5e). Imaging of endogenous hCAII in
20 live MCF7 cells, a human breast cancer cell line naturally
21 expressing hCAII, was then conducted using probe **2**. When **2**
22 was mixed with MCF7 cells followed by incubation for 4 h at
23 37°C, strong fluorescence from TMR was observed in the
24 cytosol region including the nucleus by CLSM (Figure 4d).
25 The strong fluorescence inside the cells dramatically de-
26 creased upon addition of ethoxazolamide (EZA) (Figure 4e),
27 an hCAII inhibitor,³⁰ implying that the selective ligand-hCAII
28 recognition was crucial for the turn-on imaging, similar to the
29 above-mentioned probe **1**.³¹ These results demonstrated that an
30 endogenously expressed intracellular protein can be visualized
31 by our disassembly-driven turn-on probe.³²

32 Owing to the reversible properties in living cells, a unique
33 imaging-based inhibitor screening was next constructed using
34 **2**. After staining with **2**, the MCF7 cells were incubated with

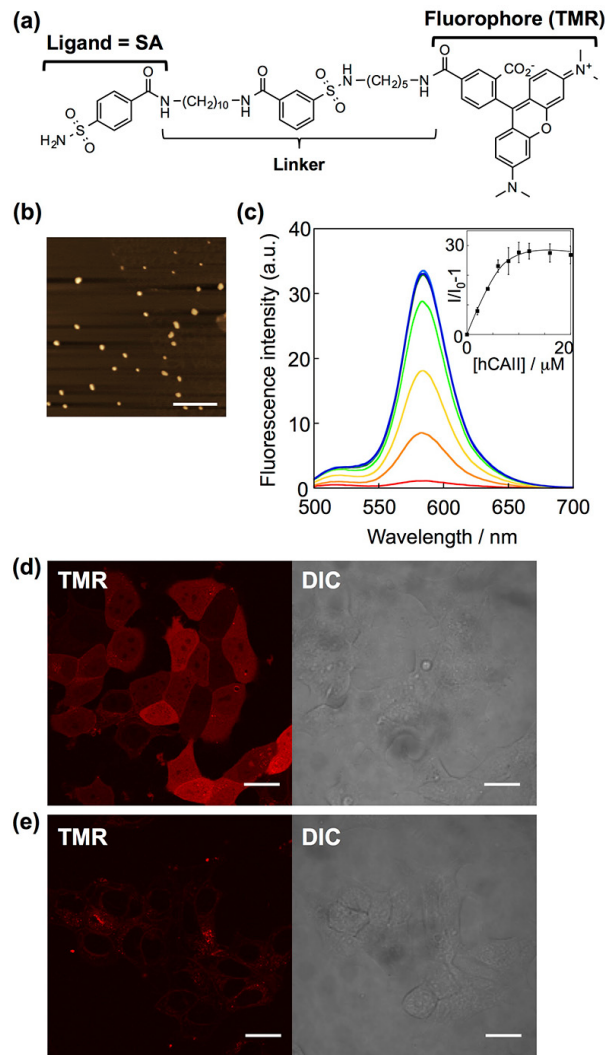


Figure 4. Endogenously expressed intracellular hCAII imaging by self-assembling nanoaggregates. (a) Chemical structure of ligand-tethered probe **2** for intracellular endogenous hCAII imaging. (b) AFM image of probe **2** (10 μ M) in 50 mM HEPES buffer (pH 7.2, 150 mM NaCl). (c) Fluorescence spectra change of probe **2** (10 μ M) upon addition of purified hCAII (0–20 μ M) (λ_{ex} = 480 nm) in 50 mM HEPES buffer (pH 7.2, 150 mM NaCl). (Inset) The relative fluorescence intensity change was plotted with the concentration of hCAII. The experiments were performed in triplicate to obtain mean and standard deviation values (shown as error bars). (d) CLSM images of MCF7 cells treated with probe **2** (300 nM) for 4 h at 37°C. The images were obtained without any washing (scale bars, 20 μ m). (e) CLSM images of MCF7 cells treated with probe **2** (300 nM) for 4 h at 37°C, and then treated with EZA (100 μ M). The images were obtained without any washing (scale bars, 20 μ m).

35 an hCAII inhibitor such as EZA, acetazolamide (AAZ) or 4-
36 sulfamoylbenzoic acid (SBA), followed by fluorescence moni-
37 toring by CLSM. When the strong inhibitor EZA was added, a
38 significant decrease in the fluorescent intensity of the intracel-
39 lular region was induced, which saturated in the nM concen-
40 tration range as shown in Figure 5a and Figure S6. In contrast,
41 no substantial change was observed by addition of 0.1 mM of
42 *p*-toluenesulfonic acid (TSA), which has negligible affinity for
43 the CA family (Figure 5f and Figure S9). We prepared the
44 titration curve on the basis of the CLSM imaging changes,
45 giving IC₅₀ values (half maximal inhibitory concentration of
46 fluorescence intensity change) of 9.9×10^{-9} M for EZA, $2.7 \times$

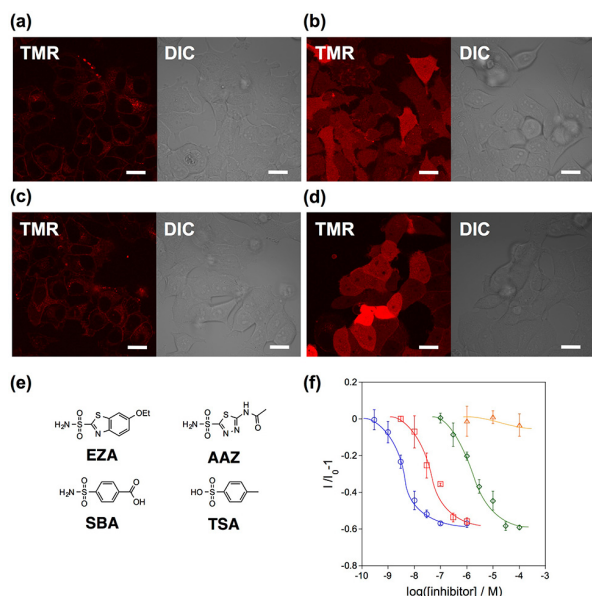


Figure 5. Imaging-based hCAII inhibitor assay with probe **2**. (a-d) Fluorescence images of MCF7 cells treated with probe **2** (100 nM), and then the cells were treated with hCAII inhibitors [(a) EZA (10 nM), (b) AAZ (10 nM), (c) AAZ (300 nM) and (d) SBA (1 μM)]. The images were obtained without any washing operations (scale bars, 20 μm). (e) Chemical structures of inhibitors for hCAII used in this study. EZA is 6-ethoxy-2-benzothiazolesulfonamide, AAZ is acetazolamide, SBA is 4-sulfamoylbenzoic acid, and TSA is 4-toluenesulfonic acid. (f) Fluorescence titration profile of the relative change in fluorescence intensity ($I/I_0 - 1$) from the intracellular region vs inhibitor concentration for EZA (○, blue), AAZ (□, red), SBA (◇, green) and non-inhibitor TSA (△, orange). Fluorescence intensities were evaluated at 10 cells in 5 regions in each dish, and the experiments were performed in triplicate to obtain mean and standard error values (shown as error bars).

1 10^{-8} M for AAZ and 1.3×10^{-6} M for SBA, respectively (Figure 5b, c, d, f and S6–S8). The literature values (K_i) determined by using purified hCAII were reported to be 8×10^{-9} M (EZA), 1.2×10^{-8} M (AAZ) and 2.6×10^{-7} M (SBA).^{30,33} While these K_i values for EZA or AAZ were almost the same as those obtained from our cell-based assay, the value for SBA was clearly different. This may be attributed to the fact that our method evaluated the lower cell-permeability and/or the lower in-cell selectivity of SBA, together with the simple affinity to CA, implying that the present cell-based inhibitor assays allow us to estimate the drug potencies under more real conditions. More importantly, these results strongly suggested that the reversible self-assembly/disassembly phenomenon of the supramolecular probes is powerful for sensing dynamic ligand/protein interactions, an essential function of endogenous proteins, even in the interior of living cells.

17 The flow cytometry analyses quantitatively supported our data from the cell imaging-based assay. The strong fluorescence was clearly observed from MCF7 cells stained with probe **2** and this fluorescence significantly decreased by addition of EZA (Figure S10a). The detailed titration curve was almost comparable to the CLSM analyses shown in Figure S10b. These results also implied that our reversible self-assembling probes can be used for the flow cytometer-based inhibitor screening systems for intracellular proteins, as well as the imaging-based assay.

27 Development of nanoaggregates for sensing of endogenous HSP90 and in-cell construction of anti-cancer agent screening system.

30 Benefiting from the modular design, a self-assembling fluorescence turn-on probe was constructed by replacing the ligand and part for another endogenously expressed intracellular protein, HSP90, a well-known tumor biomarker and valuable drug target for cancer diagnostics and treatment.^{34,35} TMR-tethered probe **3** ($\lambda_{em} = 585$ nm) carrying PU-H71 as a ligand, a selective inhibitor of HSP90, was designed (Figure 6a).^{36,37} It showed a 32-fold increase in fluorescent intensity by addition of the N-terminal ATP-binding domain of HSP90 (HSP90-N) (Figure 6b–d and Figure S11).^{38,39} The CAC value of **3** was lower than 110 nM (Figure S11b). The detection limit of **3** for HSP90-N was 70 nM (Figure S11d). We then sought to fluorescently image endogenous HSP90 in live SK-BR-3 cells, a human breast cancer cell line,^{40,41} using probe **3**. When probe **3** was mixed with SK-BR-3 cells, strong TMR fluorescence was detected in the cytosol area of SK-BR-3 cells by CLSM observation (Figure 6e). This image was almost identical to that of the immunostaining pattern obtained using an anti-HSP90 antibody (Figure S12a). The fluorescence intensity inside the cells was substantially decreased upon addition of PU-H71 (Figure 6f), suggesting that **3** was kicked out of the HSP90 binding pocket and re-assembled into nano-aggregates, similar to probe **1** (for over-expressed eDHFR) and **2** (for endogenous hCAII).⁴² Like the case of probe **2**, the flow cytometry analyses supported the results obtained by our cell image-based intensity analyses for the SK-BR-3 cells with probe **3** and PU-H71 (Figure S12c). Additionally, another turn-on probe **4**, which emitted a distinct color ($\lambda_{em} = 530$ nm) by replacing TMR with rhodamine-green (RhoG), can also be designed. This probe showed 350-fold fluorescent enhancement in response to HSP90-N (Figure S13) in test tube settings. By using probe **4**, an almost identical imaging pattern was obtained compared to probe **3** (Figure S14a).⁴³ These results clearly indicated that our self-assembling probe is capable of sensing endogenous intracellular proteins with varied colors (at least green and red colors), and these properties are good compatible with existing intracellular imaging technologies such as genetically-encoded fluorescent protein tags.

68 The titration curves for several HSP90 inhibitors were obtained, allowing the determination of the IC₅₀ values 2.1×10^{-6} M for PU-H71 and 8.1×10^{-6} M for MPC3100 (Figure 6h). In contrast, no substantial change was observed upon addition of 10 μM of geldanamycin, another inhibitor for HSP90. To date, various HSP90 inhibitors have been developed as anti-cancer agents, the potencies of which (typically as IC₅₀ values) were examined by an indirect phenotype assay, such as via tumor cell growth inhibition (the reported values are 5×10^{-8} M for PU-H71,³⁶ 6×10^{-8} M for MPC-3100⁴⁴ and 2×10^{-9} M for geldanamycin,⁴⁵ respectively). To the best of our knowledge, the results described herein provide affinity values for HSP90 inhibitors toward active HSP90 in living cells for the first time. Ultimately, this methodology should prove helpful for direct drug discovery applications in the future. Moreover, it is likely that these reversible self-assembling probes can be adapted for use in fluorescently visualizing a number of intracellular protein functions by varying the ligand and fluorophore.

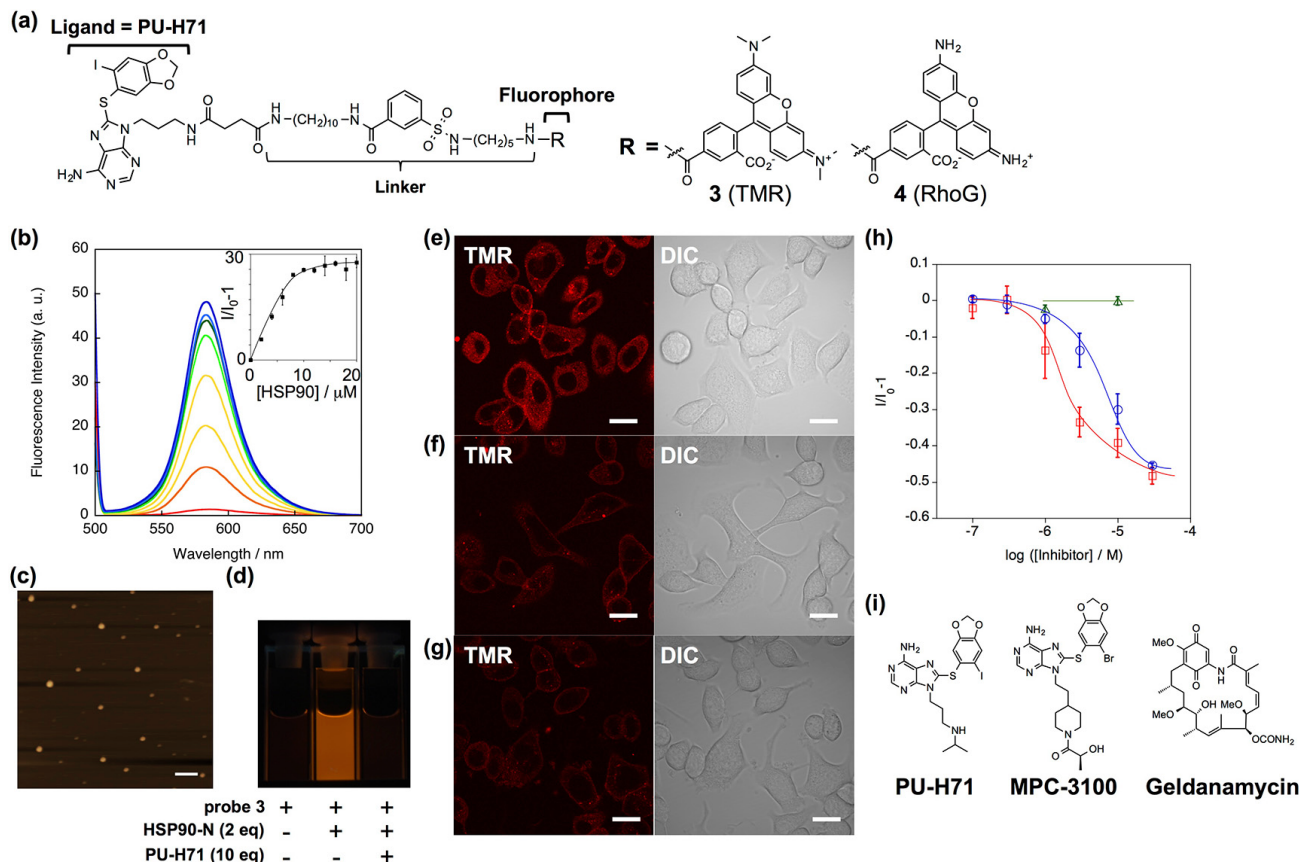


Figure 6. Endogenously expressed intracellular HSP90 imaging and inhibitor assay in living cells by self-assembling nanoaggregates. (a) Chemical structures of ligand-tethered probes **3** and **4** for intracellular endogenous HSP90 imaging. (b) Fluorescence spectra change of probe **3** (10 μM) upon addition of purified HSP90-N (0-20 μM) (λ_{ex} = 480 nm) in 50 mM HEPES buffer. (Inset) Fluorescence titration curve of probe **3** upon addition of HSP90-N (0-20 μM) (λ_{em} = 585 nm). (c) AFM image of probe **3** (10 μM) in 50 mM HEPES buffer. (d) Photographs of probe **3** (10 μM) in the absence or presence of HSP90-N (20 μM , left or middle, respectively), and after addition of PU-H71 (100 μM) to the solution of **3** and HSP90-N (right). (e) CLSM images of SK-BR-3 cells treated with probe **3** (100 nM) for 4 h at 37°C in cultured media. The images were obtained without any washing (scale bars, 20 μm). (f, g) CLSM images of SK-BR-3 cells treated with probe **3** (100 nM) for 4 h at 37°C, and then treated with PU-H71 (f, 10 μM) or MPC3100 (g, 30 μM). The images were obtained without any washing (scale bars, 20 μm). (h) Fluorescence titration profile of the relative change in fluorescence intensity ($I/I_0 - 1$) from the intracellular region vs inhibitor concentration for PU-H71 ($\square$, red), MPC-3100 ($\circ$, blue) and Geldanamycin ($\triangle$, green). Fluorescence intensities were evaluated at 10 cells in 5 regions in each dish, and the experiments were performed in triplicate to obtain mean and standard error values (shown as error bars). (i) Chemical structures of HSP90 inhibitors used in this study.

1 CONCLUSION

2 In summary, the results indicate that our protein-responsive
3 self-assembling nanoaggregates is applicable for selective and
4 reversible sensing of endogenous proteins even inside living
5 cells. The quantitative study using an overexpressed model
6 protein revealed that the designed probes form self-assembling
7 nanoaggregates in the signal-off mode in living cells in the
8 absence of the target protein, whereas the disassembly-driven
9 turn-on fluorescent change is induced in the presence of the
10 target by a specific ligand-protein recognition process. Inter-
11 estingly, the addition of a competitive inhibitor induced the
12 probe to re-assemble so that the fluorescence was turned off,
13 explicitly verifying that the reversible assembly/disassembly
14 of these supramolecular nanomaterials took place even inside
15 the living cells. The intracellular reversibility of the collapse
16 and formation of our self-assembled nanoaggregates is un-
17 precedented. This dynamic sensing mechanism allowed us to
18 construct a quantitative cell-based inhibitor assay for intracel-
19 lular endogenous proteins such as hCAII and HSP90, two val-
20 uable diagnostic and therapeutic protein targets. This work
21 should inspire new research into supramolecular approaches,
22 which work inside cells in a programmable manner for cell-

23 selective tissue engineering,⁴⁶ intracellular protein-triggered
24 drug release, or cell signaling modulation.

25 EXPERIMENTAL SECTION

26 **Synthesis.** The synthetic procedures used to prepare com-
27 pounds **1'**, **2-4** and characterizations of these compounds are
28 described in the Supporting Information.

29 **General materials and methods.** Purified human carbonic
30 anhydrase II (hCAII) was purchased from Sigma, and *Esche-*
31 *richia coli* DHFR was prepared and purified as previously
32 reported.⁴⁷ Other chemical reagents and solvents were pur-
33 chased from commercial chemical suppliers (Sigma, Aldrich,
34 TCI, Wako, or Watanabe Chemical Industries) and used with-
35 out further purification. ¹H NMR spectra were recorded on a
36 Varian Mercury-400 spectrometer (400 MHz). High-resolution
37 electrospray ionization Fourier transform mass spectrometry
38 (HR-ESI-MS) spectra were recorded using a Thermo Scien-
39 tific Exactive mass spectrometer. Cell imaging was performed
40 with a confocal laser scanning microscope (CLSM, Olympus,
41 FV1000, IX81) equipped with a 60 $\times$, NA = 1.35 oil objective
42 lens. Fluorescence images were acquired using the 488-nm
43 line of an argon laser to excite EGFP (emission, 500–530 nm),
44 the 515-nm line of an argon laser to excite Rho-G (emission,

1 530–630 nm) and the 543-nm line of a HeNe Green laser to
2 excite tetramethylrhodamine (emission, 555–655 nm). HeLa-
3 DG cells, normal Hela cells, and MCF-7 cells were maintained
4 in DMEM supplemented with 10% fetal bovine serum (FBS),
5 penicillin (100 units/mL), and streptomycin (100 µg/ mL) at
6 37°C in 5% CO₂ and 95% air. SK-BR-3 cells were maintained
7 in McCoy's 5A Modified Media supplemented with 10% FBS,
8 penicillin (100 units/mL), and streptomycin (100 µg/ mL) at
9 37°C in 5% CO₂ and 95% air. The cells were plated at a densi-
10 ty of 2.0×10^5 cells per a 35-mm glass-bottomed dish and
11 cultured in the medium for 24 h at 37°C in 5% CO₂ and 95%
12 air and used for fluorescence imaging.

13 **UV-Visible Absorption and Fluorescence Spectroscopic**
14 **Analyses.** All probes were dissolved in dimethyl sulfoxide
15 (DMSO) to generate stock solutions. The concentrations of
16 probes **1**, **2**, **3**, and **4** were determined by their absorbance (at
17 543 nm for **1**, **2** and **3**, 504 nm for **4**) in methanol using a
18 molar extinction coefficient of $92,000 \text{ M}^{-1} \text{ cm}^{-1}$ for **1**, **2** and **3**,
19 and $78,000 \text{ M}^{-1} \text{ cm}^{-1}$ for **4**,⁴⁸ respectively. HCAII was dis-
20 solved in PBS buffer (pH 7.2), and HSP90-N was dissolved in
21 10 mM Tris buffer (pH 7.4). The concentrations of these pro-
22 teins were determined by their absorbance at 280 nm using
23 literature molar extinction coefficients of $54,000 \text{ M}^{-1} \text{ cm}^{-1}$ for
24 hCAII⁴⁹ and $31,100 \text{ M}^{-1} \text{ cm}^{-1}$ for DHFR⁵⁰ respectively, and
25 $27,000 \text{ M}^{-1} \text{ cm}^{-1}$ for Hsp90-N, which was determined by its
26 absorption spectra and BCA protein assay kit allowing stock
27 solutions of known concentrations to be prepared. All experi-
28 ments were performed at 25°C in test tubes. UV-visible ab-
29 sorption spectra were recorded on a Shimadzu UV-visible
30 2550 spectrometer. Fluorescence spectra were measured using
31 a Perkin-Elmer LS55 fluorescent spectrometer. Absorption
32 and fluorescence measurements were performed 30 min after
33 adding the protein to each probe solution (10 µM stock solu-
34 tion).

35 **Fluorescence Imaging of eDHFR with probe 1.** Hela-DG
36 cells and normal Hela cells cultured in DMEM were rinsed
37 with serum- and phenol red-free DMEM supplemented with
38 100 U/mL penicillin and 100 µg/mL streptomycin (DMEM_i, 1
39 mL), and treated with probe **1** (2 µM, <1% DMSO (v/v)) in
40 DMEM_i (1 mL). Fluorescence imaging was carried out 2, 4, 6,
41 and 12 h after staining without performing any washing. For
42 the control experiments, the cells were treated with probe **1** (2
43 µM) for 12 h at 37°C, and then TMP was added (10 µM, <1%
44 DMSO (v/v)), and the fluorescence images were obtained
45 without performing any washing. For examination of the ef-
46 fects of endocytosis inhibitors, cells were treated with probe **1**
47 only (2 µM, <1% DMSO (v/v)), probe **1** (2 µM) and genistein
48 (100 µM), **1** (2 µM) and EIPA (40 µM) or **1** (2 µM) and aman-
49 tadine (500 µM). After 4 h incubation of these compounds,
50 fluorescence imaging was carried out without performing any
51 washing.

52 **Fluorescence Imaging in MCF7 and SK-BR-3 cells with**
53 **probe 2-4.** MCF7 cells plated on a 35-mm glass-bottomed
54 dish were rinsed with DMEM_i, and treated with probe **2** (300
55 nM, <1% DMSO (v/v)) in DMEM_i (1 mL). For the control
56 experiments, after incubation for 4 h, a stock solution of EZA
57 was added (final concentration, 100 µM) to the cells. Fluores-
58 cence images were obtained without performing any washing.
59 SK-BR-3 cells plated on the 35-mm glass-bottomed dish were
60 rinsed with DMEM_i, and treated with probe **3** or **4** (100 nM,
61 <1% DMSO (v/v)) in DMEM_i (1 mL). After 4 h incubation of

62 these compounds, fluorescence imaging was carried out with-
63 out performing any washing.

64 **Inhibitor assay for hCAII in MCF7 cells.** Probe **2** (100
65 nM) was added to the MCF-7 cells plated on a 35-mm glass-
66 bottomed dish. After incubation for 4 h, a stock solution of
67 each inhibitor was added to the cells. After an additional incu-
68 bation for 1 h, fluorescence imaging was carried out without
69 performing any washing. To determine the fluorescence inten-
70 sity in the cell, CLSM images were analyzed with ImageJ 1.44
71 on a Macintosh PC.

72 ASSOCIATED CONTENT

73 **Supporting Information.** Figures, experimental details and syn-
74 thetic procedures. This material is available free of charge via the
75 Internet at <http://pubs.acs.org>.

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80 Notes

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94 REFERENCES

- 95 (1) Giepmans, B. N. G.; Adams, S. R.; Ellisman, M. H.; Tsien, R. Y.
96 *Science*, **2006**, *312*, 217–224.
- 97 (2) Kobayashi, H.; Ogawa, M.; Alford, R.; Choyke, P. L.; Urano, Y.
98 *Chem. Rev.* **2010**, *110*, 2620–2640.
- 99 (3) Zhang, H.; Fan, J.; Wang, J.; Zhang, S.; Dou, B.; Peng, X. *J. Am.*
100 *Chem. Soc.* **2013**, *135*, 11663–11669.
- 101 (4) Tang, R.; Kim, C. S.; Solfiell, D. J.; Rana, S.; Mout, R.; Veláz-
102 quez-Delgado, E. M.; Chompoosor, A.; Jeong, Y.; Yan, B.; Zhu,
103 Z.-J.; Kim, C.; Hardy, J. A.; Rotello, V. M. *ACS Nano*, **2013**, *7*,
104 6667–6673.
- 105 (5) (a) Azagarsamy, M. A.; Yesilyurt, V.; Thayumanavan, S. *J. Am.*
106 *Chem. Soc.* **2010**, *132*, 4550–4551. (b) Guo, J.; Zhuang, J.; Wang,
107 F.; Raghupathi, K. R.; Thayumanavan, S. *J. Am. Chem. Soc.* **2014**,
108 *136*, 2220–2223.
- 109 (6) You, C.-C.; Miranda, O. R.; Gider, B.; Ghosh, P. S.; Kim, I.-B.;
110 Erdogan, B.; Krovi, S. A.; Bunz, U. H. F.; Rotello, V. M. *Nat.*
111 *Nanotechnol.* **2007**, *2*, 318–323.
- 112 (7) Cabral, H.; Nishiyama, N.; Kataoka, K. *Acc. Chem. Res.* **2011**, *44*,
113 999–1008.
- 114 (8) Sapsford, K. E.; Algar, W. R.; Berti, L.; Gemmill, K. B.; Casey, B.
115 J.; Oh, E.; Stewart, M. H.; Medintz, I. L. *Chem. Rev.* **2013**, *113*,
116 1904–2074.
- 117 (9) Liang, G.; Ren, H. J.; Rao, J. H. *Nat. Chem.* **2010**, *2*, 54–60.
- 118 (10) Ye, D.; Shuhendler, A. J.; Cui, L.; Tong, L.; Tee, S. S.; Tikho-
119 mirov, G.; Felsher, D. W.; Rao, J. *Nat. Chem.* **2014**, *6*, 519–526.
- 120 (11) Gao, Y.; Shi, J. F.; Yuan, D.; Xu, B. *Nat. Commun.* **2012**, *3*,
121 DOI: 10.1038/ncomms2040.

- (12) Li, J.; Gao, Y.; Kuang, Y.; Shi, J.; Du, X.; Zhou, J.; Wang, H.; Yang, Z.; Xu, B. *J. Am. Chem. Soc.* **2013**, *135*, 9907–9914.
- (13) Ding, D.; Kai, L. K.; Liu, B.; Tang, B. Z. *Acc. Chem. Res.* **2013**, *46*, 244–2453.
- (14) Shi, H.; Liu, J.; Geng, J.; Tang, B. Z.; Liu, B. *J. Am. Chem. Soc.* **2012**, *134*, 9569–9572.
- (15) Shi, H.; Kwok, R. T. K.; Liu, J.; Xing, B.; Tang, B. Z.; Liu, B. *J. Am. Chem. Soc.* **2012**, *134*, 17972–17981.
- (16) Takaoka, Y.; Sakamoto, T.; Tsukiji, S.; Narazaki, M.; Matsuda, T.; Tochio, H.; Shirakawa, M.; Hamachi, I. *Nat. Chem.* **2009**, *1*, 557–561.
- (17) Takaoka, Y.; Kiminami, K.; Mizusawa, K.; Matsuo, K.; Narazaki, M.; Matsuda, T.; Hamachi, I. *J. Am. Chem. Soc.* **2011**, *133*, 11725–11731.
- (18) Mizusawa, K.; Ishida, Y.; Takaoka, Y.; Miyagawa, M.; Tsukiji, S.; Hamachi, I. *J. Am. Chem. Soc.* **2010**, *132*, 7291–7293.
- (19) Mizusawa, K.; Takaoka, Y.; Hamachi, I. *J. Am. Chem. Soc.* **2012**, *134*, 13386–13395.
- (20) Ishida, M.; Watanabe, H.; Takigawa, K.; Kurishita, Y.; Oki, C.; Nakamura, A.; Hamachi, I.; Tsukiji, S. *J. Am. Chem. Soc.* **2013**, *135*, 12684–12689.
- (21) Baccanari, D. P.; Daluge, S.; King, R. W. *Biochemistry* **1982**, *21*, 5068–5075.
- (22) Miller, L. W.; Cai, Y.; Sheetz, M. P.; Cornish, V. W. *Nat. Methods* **2005**, *2*, 255–257.
- (23) From the intra- and extracellular fluorescent intensity in CLSM images in Figure S3e, the intracellular concentration of probe 1^{*} was roughly estimated.
- (24) Parton, R. G.; Joggerst, B.; Simons, K. *J. Cell Biol.* **1994**, *127*, 1199–1215.
- (25) Swanson, J. A.; Watts, C. *Trends Cell Biol.* **1995**, *5*, 424–428.
- (26) Meier, O.; Boucke, K.; Hammer, S. V.; Keller, S.; Stidwill, R. P.; Hemmi, S.; Greber, U. F. *J. Cell Biol.* **2002**, *158*, 1119–1131.
- (27) Phonphok, Y.; Rosenthal, K. S. *FEBS Lett.* **1991**, *281*, 188–190.
- (28) Petros, R. A.; DeSimone, J. M. *Nat. Rev. Drug Discov.* **2010**, *9*, 615–627.
- (29) Nakata, E.; Yukimachi, Y.; Nazumi, Y.; Uwate, M.; Maseda, H.; Uto, Y.; Hashimoto, T.; Okamoto, Y.; Hori, H.; Morii, T. *RSC Adv.* **2014**, *4*, 348–357.
- (30) Supuran, C. T. *Nat. Rev. Drug Discov.* **2008**, *7*, 168–181.
- (31) The quantitative comparison between the intra- and extracellular fluorescent intensity in CLSM images revealed that the probe 2 (extracellular concentration was 100 nM) was concentrated into the cell inside by 6.7-fold even in the presence of EZA (1 μ M), which enabled us to roughly estimate the intracellular concentration of probe 2 to be at least 670 ± 220 nM (Figure S5e). This value was higher than the CAC of 2 (110 nM) determined by the test tube experiments. Thus, it is conceivable indicated that the nanoprobe 2 could be self-assembled in the cell interior.
- (32) Under these conditions, almost no cytotoxicity was observed (Figure S5g).
- (33) Casini, A.; Abbate, F.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2759–2763.
- (34) Trepel, J.; Mollapour, M.; Giaccone, G.; Neckers, L. *Nat. Rev. Cancer.* **2010**, *10*, 537–549.
- (35) Chiosis, G.; Neckers, L. *ACS Chem. Biol.* **2006**, *1*, 279.
- (36) He, H.; Zatorska, D.; Kim, J.; Aguirre, J.; Llauger, L.; She, Y.; Wu, N.; Immormino, R. M.; Grewirth, D. T.; Chiosis, G. *J. Med. Chem.* **2006**, *49*, 381–390.
- (37) Modi, S.; Stopeck, A.; Linden, H.; Solit, D.; Chandarlaaty, S.; Rosen, N.; D'Andrea, G.; Dickler, M.; Moynahan, M. E.; Sugarman, S.; Ma, W.; Patil, S.; Norton, L.; Hannah, A. L.; Hudis, C. *Clin. Cancer Res.* **2011**, *17*, 5132–5139.
- (38) Prodromou, C.; Piper, P. W.; Peal, L. H. *Proteins* **1996**, *25*, 517–522.
- (39) Maroney, A. C.; Marugan, J. J.; Mezzasalma, T. M.; Barnakov, A. N.; Garrabrant, T. A.; Weaner, L. E.; Jones, W. J.; Barnakova, L. A.; Koblish, H. K.; Todd, M. J.; Mascussi, J. A.; Deckman, I. C.; Galemno, Jr. R. A.; Johnson, D. L. *Biochemistry* **2006**, *45*, 5678–5685.
- (40) Chiosis, G.; Timaul, M. N.; Munster, P. N.; Zheng, F. F.; Sepp-Lorenzino, L.; Rosen, N. *Chem. Biol.* **2011**, *8*, 289–299.
- (41) Taldone, T.; Gomes-DaGama, E. M.; Zong, H.; Sen, Siddhartha, Alpaugh, M. L.; Zatorska, D.; Alonso-Sabadell, R.; Guzman, M. L.; Chiosis, G. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 5347–5352.
- (42) Similar to probe 2, the intra- and extracellular fluorescent intensity in CLSM images showed that the intracellular concentration of probe 3 (extracellular concentration was 100 nM) was roughly estimated to be at least 1.1 ± 0.3 μ M even in the presence of PU-H71 (10 μ M) (Figure S11b). This value was higher than the CAC of 3 (110 nM) determined by the test tube experiments. Thus, it is conceivable indicated that the nanoprobe 3 could be self-assembled in the cells interior.
- (43) Similar to probes 1 and 2, almost no cytotoxicity was observed both in cases of probes 3 and 4 (Figure S12d and S14d).
- (44) Kim, S.-H.; Bajji, A.; Tangallapally, R.; Markovitz, B.; Trovato, R.; Shenderovich, M.; Baichwal, V.; Bartel, P.; Cimbora, D.; McKinnon, R.; Robinson, R.; Panac, D.; Wettstein, D.; Carlson, R.; Yager, K. M. *J. Med. Chem.* **2012**, *55*, 7480–7501.
- (45) Hartmann, F.; Horak, E. M.; Cho, C.; Lupu, R.; Bolen, J. B.; Steler-Stevenson, M. A.; Pfreundschuh, M.; Waldmann, T. A.; Horak, I. D. *Int. J. Cancer* **1997**, *70*, 221–229.
- (46) Hirata, N.; Nakagawa, M.; Fujibayashi, Y.; Yamauchi, K.; Murata, A.; Minami, I.; Tomioka, M.; Kondo, T.; Kuo, T.-F.; Endo, H.; Inoue, H.; Sato, S.; Ando, S.; Kawazoe, Y.; Aiba, K.; Nagata, K.; Kawase, E.; Chang, Y.-T.; Suemori, H.; Eto, K.; Nakauchi, H.; Yamanaka, S.; Nakatsuji, N.; Ueda, K.; Uesugi, M. *Cell Reports* **2014**, *6*, 1165–1174.
- (47) Tanaka, T.; Kamiya, N.; Nagamune, T. *FEBS Lett.* **2005**, *579*, 2092–2096.
- (48) Haugland, R. P. The handbook: a guide to fluorescent probes and labeling technologies, 10th ed.; Invitrogen: Carlsbad, CA, **2005**.
- (49) Supuran, C. T.; Briganti, F.; Tilli, S.; Chegwiddden, W. R.; Scozzafava, A. *Bioorg. Med. Chem.* **2001**, *9*, 703–714.
- (50) Touchette, N. A.; Perry, K. M.; Matthews, C. R. *Biochemistry* **1986**, *25*, 5445–5452.

