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MCM−Enzyme−Supramolecular Hydrogel Hybrid as a Fluorescence Sensing Material for Polyanions of Biological Significance

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Abstract: Polyanions are important sensing targets because of their wide variety of biological activities. We report a novel polyanion-selective fluorescence sensing system composed of a hybrid material of supramolecular hydrogel, enzymes, and aminoethyl-modified MCM41-type mesoporous silica particles (NH_2−MCM41) encapsulating anionic fluorescent dyes. The rational combination of the polyanion-exchange ability of NH_2−MCM41 and semi-wet supramolecular hydrogel matrix successfully produced three distinct domains; namely, cationic nanopores, hydrophobic nano/microfibers, and aqueous bulk gel phase, which are orthogonal to each other. The coupling of anion-selective probe release from NH_2−MCM41 with translocation of the probe facilitated by enzymatic reaction enabled fluorescence resonance energy transfer-type sensing in the hybrid materials for polyanions such as heparin, chondroitin sulfate, sucrose octasulfate, and so forth. The enzymatic dephosphorylation catalyzed by phosphatase (alkaline phosphatase or acid phosphatase) that is embedded in gel matrix with retention of activity also contributed to improving the sensing selectivity toward polysulfates relative to polyphosphates. It is clear that the orthogonal domain formation of these materials and maintaining the mobility of the fluorescent dyes between the three domains are crucial for the rapid and convenient sensing provided by this system.

Introduction

Polyanions such as polysulfates, including heparin, chondroitin sulfate, and sucrose octasulfate (Suc-8S), and polyphosphates, including adenosine triphosphate (ATP), 1,4,5-inositol triphosphate (IP_3), inositol hexaphosphate (IP_6), phosphorylated proteins, polynucleotides such as DNA and RNA, and phospholipids, are ubiquitous in nature. They all express a variety of biological activities. For instance, heparin, a highly sulfated glycosaminoglycan, is commonly used as an injectable anticoagulant,[1] and chondroitin sulfate, which is another sulfated glycosaminoglycan and is found in cartilage, is widely used as a dietary supplement to treat arthritis.[2] Suc-8S has been recently reported to stimulate fibroblast growth factor (FGF) signaling through increasing FGF−FGF receptor affinity and promoting receptor dimerization, which results in wound healing by enhancing FGF-induced angiogenesis, as in the case of heparin and heparin sulfate proteoglycans.[3] Among polyphosphates, ATP is a well-known main energy source for the majority of cellular functions, and IP_3 is one of the important secondary messengers of signal transduction in live cells.[4] IP_6, which is abundant in many plant sources, has recently received much attention as nutrition against phosphate crisis[5a−c] and for its role in cancer prevention and control of tumor growth, progression, and metastasis.[5d,e] Thus, the development of rapid, convenient, and high-throughput sensing systems for such polyanions is of considerable significance. In particular, contamination by oversulfated chondroitin sulfate found in certain lots of heparin was recently reported to cause serious side effects presumably due to anaphylactoid response.[6] Simple and selective detection of such polysulfates is now in much demand; however, polysulfate-selective sensors have been poorly explored compared with sensors for polyphosphates.[7,8]

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As an example of molecular sensors for polyanions operating in solution, Anslyn's group recently succeeded in developing artificial receptors with phenylboronic acid and ammonium group, which can bind anionic polysaccharide such as heparin through boronate ester formation and electrostatic interaction.[9] Although these molecular receptors are powerful,[10] this approach often suffers from the tedious synthetic procedures and complex design required, especially for complicated sensing targets. As a unique alternative, Matile's group exploited fluorogenic sensor systems comprising molecular assemblies such as vesicles functionalized with engineered rigid-rod β barrels as the nanopore that is selectively blocked by an analyte of interest.[11] They demonstrated that the difference in the pore blockage efficiency of polyanions such as ATP and ADP can be monitored as the fluorogenic release of self-quenched fluorescent dye. Very recently, Amorós and co-workers reported that mesoporous silica particles (MCM41) bearing well-ordered nanopores, the surface of which was functionalized to be cationic, can be also utilized as colorimetric sensors on the basis of ion-exchange phenomena between encapsulated anionic dyes and simple anions such as carboxylates and phosphates.[12,13]

In contrast to these solution-based analyses, array technology immobilizing sensing devices on solid supports is now rapidly growing to afford DNA/protein/peptide/saccharide arrays for high-throughput and efficient analysis.[14] We recently proposed a chemosensor array in which supramolecular hydrogels were employed as a semi-wet matrix for noncovalently immobilizing a variety of chemosensors.[15,16] Here, we describe a fluorescence sensing system consisting of a hybrid material of supramolecular hydrogel, enzyme, and MCM41-type mesoporous silica particles encapsulating a fluorescent probe in the pores. In this hybrid system, three distinct microdomains, namely the cationic nanopores, hydrophobic nano/microfibers, and aqueous gel bulk phase, were produced in semi-wet condition and are orthogonal to each other. Coupling of the anion-selective release of the probe from NH_2−MCM41 with translocation facilitated by enzymatic reaction successfully yielded a unique fluorescence resonance energy transfer (FRET)-type sensing material for polyanions such as heparin, Suc-8S, and so forth. Dephosphorylation catalyzed by enzymes embedded in the gel phase improved the discrimination ability of the sensor system toward polysulfates relative to polyphosphates. The orthogonal domain formation and sufficient mobility of the embedded molecules are crucial for such a cooperative sensing system. We also constructed an MCM41−hybrid array capable of distinguishing polyanions such as heparin, Suc-8S, and IP_6 with high throughput.

Results and Discussion

Polyanion Responsive Release of Fluorescent Probe Entrapped within Amine-Modified MCM41. It was reported that the interior of MCM41 is able to entrap anionic species by appropriate modification with cationic amines.[12,13,17] We prepared aminoethyl-modified MCM41 (NH_2−MCM41) according to the reported procedure,[17] namely, the CTAB template method using aminoethyl triethoxysilane and tetraethoxysilane (see Supporting Information). We then confirmed that it can encapsulate a phosphorylated serin appended coumarin (P-coum **2**) in acidic aqueous solution (pH 5.0, 28 ± 0.3 nmol mg^{-1} of NH_2−MCM41). It also became clear that the encapsulated P-coum **2** was released up to 88% by pH shift from 5 to 10 (Figure 1a). A detailed pH dependence study showed that the release (%) was sharply enhanced above neutral pH, and the apparent pK_a value was determined as 7.5, suggesting the crucial role of electrostatic interaction between NH_2−MCM41 and P-coum **2** for the entrapment (Figure 1b). We also confirmed that the release of P-coum **2** from NH_2−MCM41 was triggered by an anion-exchange reaction. For instance, addition of sucrose octasulfate (Suc-8S) to the suspension of NH_2−MCM41 in aqueous buffer solution facilitated the release (63%) of P-coum **2** by increasing the Suc-8S content without changing the pH (pH 5.0) (Figure 2a). It is interesting that, as shown in Figure 2b, the release was efficiently induced by other polysulfates and polyphosphates such as heparin, chondroitin sulfate, ATP, and IP_6, but not by polycarboxylate (hyaluronic acid), monoanions (AMP, sialyl lactose, and inorganic phosphate), neutral (mal-

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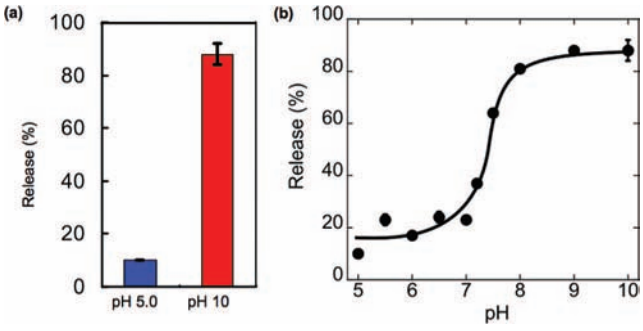

Figure 1. (a) Release (%) of P-coum **2** in 200 mM acetate buffer (pH 5.0, 600 μL) or 50 mM CHES buffer (pH 10, 600 μL) from NH_2−MCM. (b) pH-Dependent release profiles of P-coum **2** from NH_2−MCM. Conditions: [P-coum **2**@NH_2−MCM] = 0.83 mg mL^{-1} (0.50 mg), RT.

toheptaose), and cationic substrates (glucosamine). These results may be ascribed to the polyanionic molecules with phosphate or sulfate groups being more strongly bound to the cationic interior of NH_2−MCM41 than the less anionic P-coum **2**, so that **2** was replaced by the polyanions. The pore size of NH_2−MCM41, estimated as 2.7 nm from BET measurement (Supporting Information), offered additional selectivity toward anionic species. For instance, bulky polyanions such as ovalbumin and double-stranded DNAs (plasmid DNA and λ-DNA) were not able to replace P-coum **2**, suggesting that the size of the polyanions is another controlling factor for the anion-exchange in NH_2−MCM41.

Catch and Release Function of Amine-Modified MCM41 in Supramolecular Hydrogel. On the basis of the results of the polyanion-triggered fluorophore release from NH_2−MCM41, we designed a novel MCM-based fluorescence sensing device by hybridization with a unique microenvironment of supramolecular hydrogel.[18,19] Scheme 1 illustrates our strategy for the construction of the MCM−enzyme−supramolecular hydrogel hybrid sensor. We expected that anion exchange within NH_2−MCM41 took place even in the supramolecular hydrogel matrix, owing to its semi-wet environment similar to aqueous solution. By accumulation of the fluorescent probe released from NH_2−MCM41 on the hydrophobic fibers of supramolecular hydrogel, we can induce a fluorescence change within the gel matrix. Efficient translocation of the probe from the aqueous gel phase to the fiber may be facilitated by its chemical transformation catalyzed by the embedded enzymes. The altered fluorescence is a suitable readout signal that is sensitive to the character and the amount of added anions. Since we showed that NH_2−MCM41 responded selectively to polyanions in aqueous solution, a composite of NH_2−MCM41 and enzyme with supramolecular hydrogel may be a unique polyanion-sensing hybrid material.

We initially evaluated the catch and release function of NH_2−MCM41 embedded in a supramolecular hydrogel **1** matrix. Using confocal laser scanning microscopy (CLSM), we clearly observed aggregated NH_2−MCM41 in a hydrogel droplet[20] as green fluorescent spots (ca. 1−5 μm in diameter) due to the bound P-coum **2**. It was also shown that NH_2−MCM41 was segregated from the supramolecular fibers stained by the hydrophobic BODIPY dye **4**, indicating that these two materials can provide microdomains orthogonal to each other in the semi-wet matrix (Figure 3). When the pH of the gel matrix was changed from 5 to 10, the green spots became smeared, suggesting pH-triggered release of P-coum **2** from the interior of NH_2−MCM41 to the aqueous gel space. Similarly, the intensity of the green fluorescence decreased on addition of Suc-8S. In this case, we quantitatively evaluated the relative release ratio using red fluorescent microbeads (10 μm in diameter) as an internal standard. Figure 4 shows the CLSM images and the corresponding line profiles of fluorescence intensity traversing the NH_2−MCM41 and the fluorescent microbeads, before and after Suc-8S addition. Apparently, the green fluorescence due to P-coum **2** entrapped in the interior of NH_2−MCM41 decreased in its intensity by addition of Suc-8S and the intensity change ($1 - F/F_0 = 0.60 \pm 0.06$) was estimated by comparison with the red fluorescent microbeads. Figure 5 summarizes the fluorescence change caused by various substances. As in the case of aqueous suspension (Figure 2), heparin, ATP, and IP_6 were effective for releasing P-coum **2** from NH_2−MCM41, as well as Suc-8S. By contrast, AMP, inorganic phosphate, maltoheptaose, and ds-DNA were not effective. Thus, it is clear that the selective anion-exchange property of the NH_2−MCM41/P-coum **2** composite was retained even when it was embedded in the supramolecular hydrogel matrix **1**.

Polyanion Triggered and Enzyme-Coupled Translocation of Fluorescent Probe in NH_2−MCM41 Supramolecular Hydrogel Hybrid. By CLSM observation, we confirmed the release of P-coum **2** from NH_2−MCM41 in the gel matrix. Unfortunately, we also found that the P-coum **2** did not accumulate in the gel fibers, but dispersed in the aqueous space of the gel matrix, resulting in smeared CLSM images. To facilitate accumulation of the fluorescent probe to the fiber domains, we next introduced coupled enzymatic hydrolysis of the anionic phosphate group of the P-coum probe. We already reported that enzymes can be immobilized in saccharide-based supramolecular hydrogels without loss of natural activity[16] and that sufficient mobility of small molecules is retained in the gel matrix.[15,16] For efficient accumulation of the fluorescent probe in the hydrophobic fiber domains after enzymatic hydrolysis, we introduced a hydrophobic cyclohexyl group into the C-terminus of P-coum **2**, which gave a new probe P-coum **3**.[21]

We investigated the polyanion-triggered and enzyme-coupled translocation of P-coum **3** by CLSM observations. Figure 6 shows typical CLSM images of the hybrid gel before and after treatment with Suc-8S and/or acid phosphatase (ACP). While the addition of only Suc-8S simply decreased the fluorescence intensity from the spots, the green fluorescent fibers were clearly visualized by subsequent addition of ACP. Similar CLSM image changes occurred by pH shift from 5 to 10 followed by alkaline phosphatase (AP) treatment (data not shown). We also confirmed that the translocation of probe **3** never occurred without Suc-

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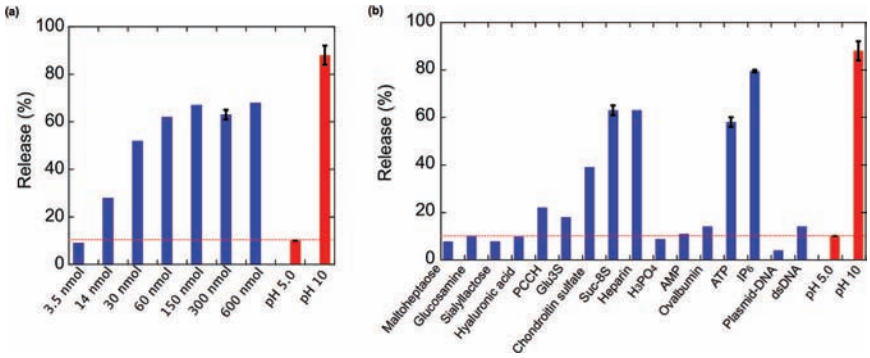
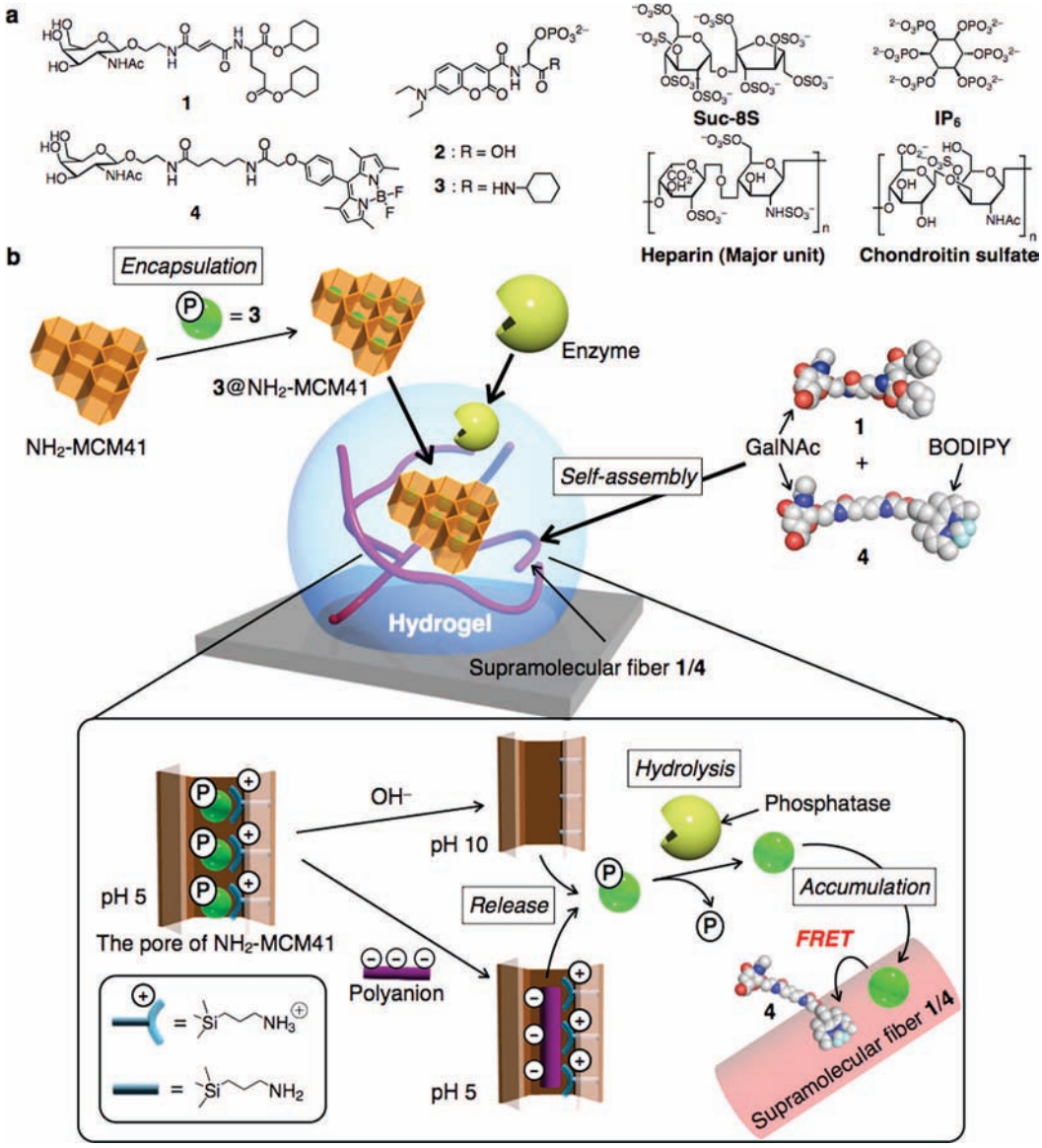


Figure 2. (a) Dependence of the release (%) of P-coum **2** from NH_2−MCM on the concentration of Suc-8S ([Suc-8S] = 0−1.0 mM (0−600 nmol)) in 200 mM acetate buffer (pH 5.0, 600 μL). (b) Release (%) of P-coum **2** from NH_2−MCM upon the addition of various anionic species in 200 mM acetate buffer (pH 5.0, 600 μL). Conditions: [anion] = 0.50 mM (300 nmol), [ovalbumin], [dsDNA (λ-DNA)] = 0.30 mM (180 nmol), [plasmid DNA] = 0.25 mM (152 nmol), [P-coum **2**@NH_2-MCM] = 0.83 mg mL^{-1} (0.50 mg), RT.

Scheme 1. (a) Chemical Structures of Compounds **1**−**4**, Suc-8S, IP_6, Heparin, and Chondroitin Sulfate and (b) Construction and Mechanism Operating in Fluorescent Dye Encapsulated MCM−Enzyme−Supramolecular Hydrogel Hybrid Sensory System



8S addition or basic pH shift, even in the presence of the phosphatase enzymes, suggesting that P-coum **3** entrapped in the interior of NH_2−MCM41 is completely protected from enzymatic hydrolysis. These results imply that the P-coum **3** released by Suc-8S or pH shift was hydrolyzed by the phosphatase (ACP or AP) in the aqueous space of the gel matrix, and subsequently the resulting neutral coumarin, which was more hydrophobic than anionic P-coum **3**, migrated to the

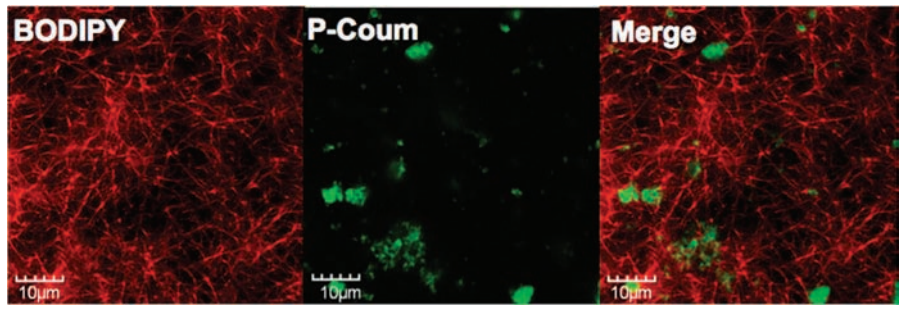


Figure 3. CLSM images of the hydrogel **1** droplet containing BODIPY **4** and P-coum **2** encapsulated NH_2−MCM. The left image shows the localization of BODIPY **4** (λ_{ex} = 488 nm/λ_{em} = 515−570 nm), the middle image shows the localization of P-coum **2** (λ_{ex} = 458 nm/λ_{em} = 470−510 nm), and the merge image shown in the right panel was obtained by summing the left and middle images. Conditions: [**1**] = 0.090 wt % (50 mM Tris-HCl buffer, pH 5.0), [**4**] = 5.0 μM, [P-coum **2**@NH_2−MCM] = 9.0 mg mL^{-1} (4.5 μg), RT.

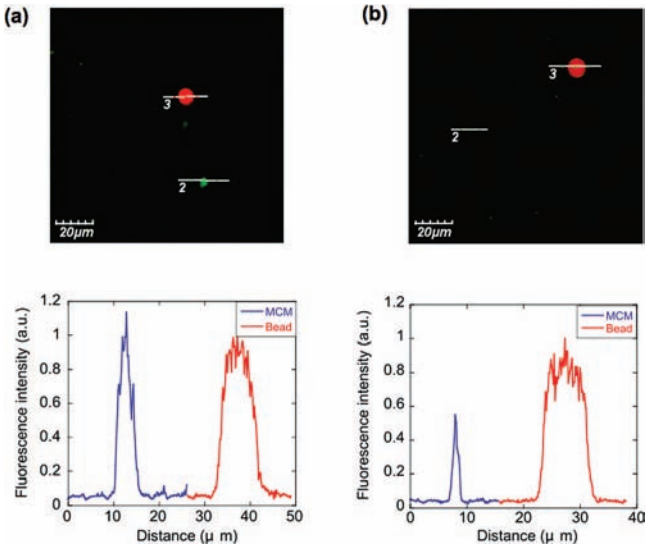


Figure 4. CLSM images of the hydrogel **1** droplet containing P-coum **2** encapsulated NH_2−MCM and 10-μm microbeads (a) before and (b) after 120 min of the Suc-8S addition. Fluorescence intensity profiles along lines 2 and 3 in each image are shown below the images. Conditions: [**1**] = 0.090 wt % (200 mM acetate buffer, pH 5.0), [Suc-8S] = 0.42 mM (0.50 nmol), [P-coum **2**@NH_2−MCM] = 0.83 mg mL^{-1} (0.80 μg), RT.

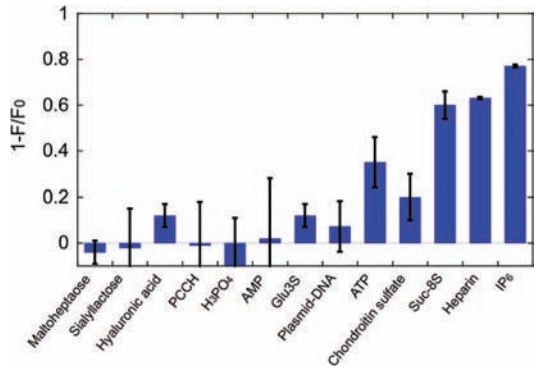


Figure 5. Changes in fluorescence intensity ratio of the hydrogel **1** droplet containing P-coum **2** encapsulated NH_2−MCM upon the addition of anionic species. Conditions: [**1**] = 0.090 wt % (200 mM acetate buffer, pH 5.0), [anion] = 1.0 mM (0.50 nmol), [P-coum **2**@NH_2−MCM] = 0.83 mg mL^{-1} (0.80 μg), RT.

hydrophobic gel fibers. It should be noted that all of these events took place within a single hydrogel droplet.

Polyanion-Selective Fluorescence Sensing by MCM41−ACP−Hydrogel Hybrid. Such translocation of the fluorescent probe within the hydrogel matrix can be readily visualized using FRET-type emission change, which provides a hydrogel-based fluorescent sensor selective to polyanions. For FRET sensing, we embedded a suitable FRET acceptor in the hydrophobic domain of the supramolecular fibers. Controlled juxtaposition of the FRET acceptor, a BODIPY derivative **4**, was easily accomplished because of the high orthogonality of the three microdomains, that is, the cationic interior of MCM41, the aqueous space of the hydrogel, and the hydrophobic fibrous domain.

Figure 7 shows CLSM images of the hybrid hydrogels containing BODIPY **4**, AP, and NH_2−MCM41 entrapping P-coum **3**. At acidic pH, the hydrophobic BODIPY stained the supramolecular fibers, whereas the P-coum **3** spots were separately located within NH_2−MCM41. Thus, FRET emission between coumarin and BODIPY was not observed. After pH shift from 5 to 10, the segregated spots due to P-coum **3** almost disappeared and the coumarin fluorescence was localized along the fibers and overlapped perfectly with the emission from BODIPY (see the merged image). Importantly, FRET emission (CLSM images under condition 3) was also obtained along the fiber domains. Thus, it is clear that the translocation of the coumarin fluorophore from the interior of NH_2−MCM41 to the supramolecular fiber via the aqueous gel space greatly facilitated the change in FRET emission.

This FRET behavior allowed us to monitor the pH-induced fluorescence spectral change of the gel spots, as shown in Figure 8a. The emission at 483 nm from coumarin decreased, and the emission intensity at 513 nm from BODIPY concurrently increased. A seesaw type of emission change also occurred by addition of Suc-8S, as shown in Figure 8b. The change was saturated with increase in the Suc-8S content (inset of Figure 8b), clearly demonstrating the validity of the hybrid hydrogel as a fluorescent anion-sensing material. Figure 8c summarizes titration curves of the hybrid gel with various substances. IP_6, Suc-8S, and heparin were detected at a 5 nmol level (1 μL of 5 mM analyte solution), whereas sialyl lactose and glucosamine were not sensed because of their poor anion-exchange activity. Also, we confirmed that Suc-8S could be detected even in the coexistence of other anions such as sialyl lactose, AMP, and so forth (Figure S4 in the Supporting Information). Interestingly, we noticed that IP_3 and ATP were scarcely detected using this hybrid hydrogel despite the high anion-exchange activity. This can probably be ascribed to ACP immobilized in the hybrid gel rapidly hydrolyzing the phosphoester bonds of IP_3 and ATP so as to reduce their concentrations before anion exchange, indicative of a masking effect of ACP for such polyphosphates. As a result, the present NH_2−MCM41−ACP hydrogel hybrid

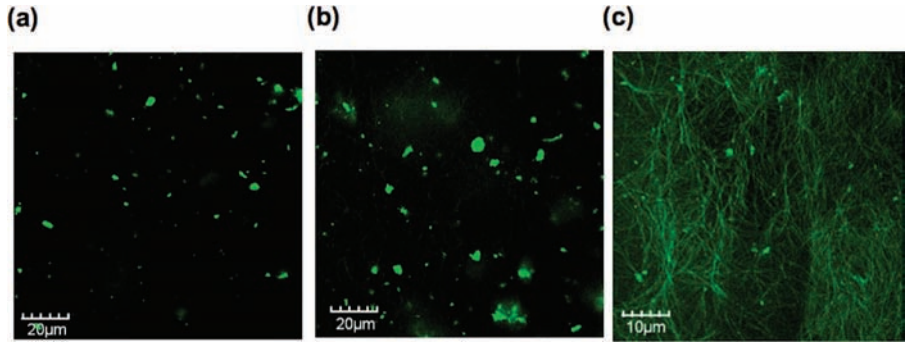


Figure 6. CLSM images (λ_{ex} = 458 nm) of hydrogel **1** containing P-coum **3** encapsulated NH_2−MCM (a) before, (b) after 60 min of Suc-8S addition ([Suc-8S] = 1.15 mM, 15 nmol), and (c) after 90 min of subsequent ACP addition. Conditions: [**1**] = 0.090 wt % (200 mM acetate buffer, pH 5.0), [P-coum **3**@NH_2−MCM] = 5.0 mg mL^{-1} (50 μg), RT.

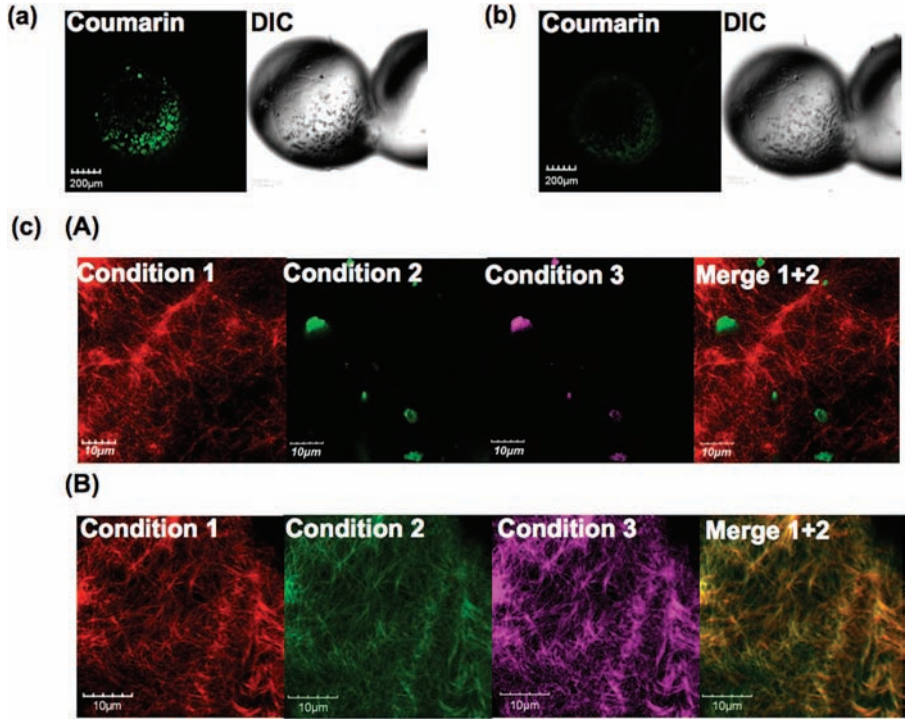


Figure 7. Low-magnification CLSM (×10 objective) images of hydrogel droplet **1** (left side) containing BODIPY **4** and P-coum **3** encapsulated NH_2−MCM in hexadecane (a) before and (b) after 60 min of adjusting to pH 10 and AP addition, which was conducted by the fusion of another hydrogel droplet **1** (right side) (left: λ_{ex} = 488 nm/λ_{em} = 515−570 nm; right: DIC). (c) CLSM (×100 objective) images of the hydrogel **1** droplet. The series of images in (A) and (B) corresponds to (a) and (b), respectively. The images of condition 1 show the localization of BODIPY **4** (λ_{ex} = 488 nm/λ_{em} = 515−570 nm), and the images of condition 2 show the localization of P-coum **3** (λ_{ex} = 458 nm/λ_{em} = 470−510 nm). The images of condition 3 mainly correspond to FRET emission from P-coum **3** to BODIPY **4** (λ_{ex} = 458 nm/λ_{em} = 515−570 nm), although these partially contain contribution of the direct emission from P-coum **3**, and the merged images shown in the right panels were obtained by summing the images of conditions 1 and 2. Conditions: [**1**] = 0.090 wt % (50 mM Tris-HCl buffer, pH 5.0), [**4**] = 2.0 μM, [P-coum **3**@NH_2-MCM] = 9.0 mg mL^{-1} (4.5 μg), RT.

showed improved selectivity for polysulfates relative to polyphosphates.

We finally prepared a hybrid material array for rapid and high-throughput sensing for a variety of substrates. As shown in Figure 9a, bright emission was observed at spots containing IP_6, heparin, Suc-8S, and chondroitin sulfate, but less bright emission came from ATP, sialyl lactose, maltopentaose, and others. Naked eye detection of polyanions was very similar to the titration curves in its selectivity, demonstrating the utility of the present hybrid materials for high-throughput polyanion sensing. Figure 9b shows a photograph of the phosphate anion-sensing chip of **1** containing coumarin-appended phosphate receptor **12** and the amphiphilic FRET acceptor (styryl dye) **13**, which was previously developed by us.[15a] Apparently, the spot colors of polyphosphates such as IP_6, IP_3, and ATP changed to bluish, but that color change was not induced by polysulfates. The color change from red to blue is due to the cancelation of FRET from the blue fluorescent coumarin dye to the red fluorescent styryl dye, which indicates that receptor **12** is released from the hydrophobic fiber **1** containing **13** upon binding to the polyphosphates.[15a] Simple comparison between these two chips by the naked eye thereby allows us to discriminate polysulfates such as heparin and Suc-8S from polyphosphates such as IP_6 and ATP.

Conclusions

We demonstrated that rational combination of the anion-exchange ability of NH_2−MCM41 and the semi-wet supramolecular hydrogel matrix successfully produced unique sensing materials selective to polyanions such as polysulfate oligosac-

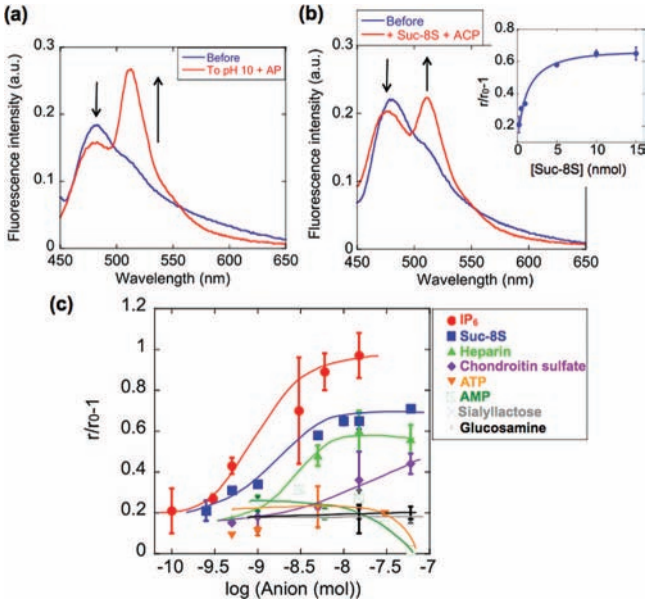


Figure 8. Fluorescence spectral changes (λ_{ex} = 429 nm) of hydrogel **1** containing BODIPY **4** and P-coum **3** encapsulated NH_2−MCM (a) before and after adjusting to pH 10 and AP addition and (b) before and after Suc-8S and ACP addition. The inset in (b) shows the fluorescence titration curve for Suc-8S ([Suc-8S] = 0−1.4 mM (0−15 nmol)). Conditions: [**1**] = 0.090 wt % (50 mM Tris-HCl buffer, pH 5.0, [**4**] = 2.0 μM for (a) and 200 mM acetate buffer, pH 5.0, [**4**] = 3.0 μM, [Suc-8S] = 1.4 mM (15 nmol) for (b)), [P-coum **3**@NH_2−MCM] = 5.0 mg mL^{-1} (50 μg), RT. (c) Plots of the relative fluorescence intensity changes ($r/r_0 - 1 = (F_{513}/F_{483})/(F_{513}/F_{483})_0 - 1$) of hydrogel **1** containing BODIPY **4** and P-coum **3** encapsulated NH_2−MCM upon addition of various species and ACP (λ_{ex} = 429 nm). Conditions: [**1**] = 0.090 wt % (200 mM acetate buffer, pH 5.0), [**4**] = 3.0 μM, [anion] = 0−6.5 mM (0−60 nmol), [P-coum **3**@NH_2−MCM] = 5.0 mg mL^{-1} (50 μg), RT.

charides. The orthogonal domain formation given by these two materials and maintaining sufficient mobility of small molecules are crucial for this sensing system. Coupling of the enzyme reaction in the hybrid gel efficiently modulates the sensing efficiency and selectivity. It is envisioned that further functionalization of the MCM and/or the gel fibers, and a new entry of various enzymes, may lead not only to sophisticated sensing materials, but also to intelligent drug delivery systems or other biomaterials. We are now working along this line.

Experimental Section

General. Unless stated otherwise, all commercial reagents were used as received. Alkaline phosphatase (from *Escherichia coli*, TOYOBO) and acid phosphatase (from *potato*, SIGMA) were used as received. Thin layer chromatography was performed on silica gel 60F_{254} (Merck). Column chromatography was performed on silica gel 60N (Kanto, 40−50 μm). Reverse-phase HPLC was conducted with a Hitachi Lachrom instrument with YMC-pack ODS-A columns. ^{1}H NMR spectra were obtained on a Varian Mercury 400 spectrometer with tetramethylsilane or residual nondeuterated solvents as the internal references. FAB, MALDI-TOF, and ESI mass spectra were recorded using a Shimadzu QP5050A (NBA as a matrix), Perseptive Biosystems Voyager DE-RP instruments (dithranol as a matrix), and Applied Biosystems API2000, respectively. The absorption and fluorescence spectra were measured using a Shimadzu UV2550 and a Perkin-Elmer LS55 spectrometer, respectively. Fluorescence spectra of the hydrogel spots on a slide glass plate were recorded using an Otsuka Electronics high-sensitivity Spectro multichannel photodetector, MCPD-7000. TEM images were obtained by using a JEOL JEM-2010 microscopy. FTIR spectra were recorded by using a Perkin-Elmer Spectrum One on ATR mode. BET nitrogen sorption experiments were carried out using a Belsorp Belsorp-18 volumetric adsorption equipment. Elemental analysis was carried out by the services at Kyoto University.

Synthesis. The gelator **1** was prepared according to the method reported previously by us.[22] P-coum **2** and **3** were prepared as shown in Schemes 2 and 3, respectively. The synthetic procedure for P-coum 2 is described in the Supporting Information. BODIPY **4** was prepared from BODIPY-carboxylic acid[23] and GalNAc-NH_2,[19] as shown in Scheme 4.

Fmoc-*O*-benzyloxy-phosphono-L-serin Cyclohexyl Amide (6). A solution of Fmoc-*O*-benzyloxy-phosphono-L-serin (300 mg, 0.60 mmol) and benzotriazol-1-yloxy-tris(dimethylamino)phosphate hexafluorophosphate (BOP, 320 mg, 0.72 mmol) in dry DMF (5 mL) was stirred at 0 °C under Ar atmosphere for 30 min. Cyclohexylamine (87 μL, 0.72 mmol) was then added to the solution. The mixture solution was stirred for 2 h, and then the solvent was removed under reduced pressure. The residue was dissolved in ethyl acetate (100 mL), and the solution was washed with aqueous sodium bicarbonate (100 mL), 5% aqueous citric acid (100 mL), and brine (100 mL). The organic layer was collected and dried over anhydrous Na_2SO_4 and filtered. The filtrate was concentrated to dryness, and the residue was purified by column chromatography (SiO_2, chloroform/methanol = 4:1), affording compound **6** as a white solid (136 mg, 39%). ^{1}H NMR (400 MHz, $CDCl_3$, room temperature): δ 1.61−1.81 (m, 10H), 3.64−3.65 (m, 1H), 4.00−4.09 (m, 2H), 4.18−4.21 (m, 1H), 4.31−4.64 (m, 1H + 2H), 4.87 (d, J = 6.0 Hz, 1H), 7.23−7.80 ppm (m, 5H + 10H); MALDI-MS obsd 579.93 $[M + H]^+$, calcd 579.22.

***O*-Benzyloxy-phosphono-L-serin Cyclohexyl Amide (7).** A solution of Fmoc-*O*-benzyloxy-phosphono-L-serin cyclohexyl amide **6** (136 mg, 0.24 mmol) in 20% piperidine DMF (8 mL) was stirred at room temperature. After 45 min, the solvent was removed under reduced pressure. The residue was subjected to precipitation by diisopropyl ether to afford compound **7** as a white solid (82 mg, 97%). ^{1}H NMR (400 MHz, CD_3OD, room temperature): δ 1.11−1.34 (m, 10H), 3.30−3.38 (m, 1H (overlapped with methanol)), 3.52−3.72 (m, 1H), 3.98−4.02 (m, 1H), 4.88−4.91 (d, 2H (overlapped with H_2O)), 7.26−7.38 ppm (m, 5H); MALDI-MS obsd 357.78 $[M + H]^+$, calcd 357.15.

Coumarin-*O*-benzyloxy-phosphono-L-serin Cyclohexyl (8). A solution of 7-diethylamino coumarin-3-carboxyric acid (35 mg, 0.16 mmol) and BOP (85 mg, 0.19 mmol) in dry DMF (3 mL) was stirred at 0 °C under Ar atmosphere for 30 min. *O*-Benzyloxy-phosphono-L-serin cyclohexy amide **7** (56 mg, 0.16 mmol) in dry DMF (5.5 mL) and DIPEA (55 μL, 0.32 mmol) was added to the solution. The mixture solution was stirred at room temperature for 4 h, and then the solvent was removed under reduced pressure. The residue was dissolved in ethyl acetate (50 mL), and the solution was washed with aqueous sodium bicarbonate (50 mL), 5% aqueous citric acid (50 mL), and brine (50 mL). The organic layer was collected and dried over anhydrous Na_2SO_4 and filtered. The filtrate was concentrated to dryness, and the residue was purified by column chromatography (SiO_2, chloroform/methanol = 4:1), affording compound **8** as a yellow solid (38 mg, 40%). ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$ = 6:1, room temperature): δ 1.13−1.88 (m, 10H), 1.23 (t, J = 7.2 Hz, 6H), 3.44 (q, J = 7.2 Hz, 4H), 3.65−3.74 (m, 1H), 4.19−4.20 (m, 2H), 4.86−4.87 (m, 1H), 4.97 (d, J = 4.8 Hz, 2H), 6.40 (s, 1H), 6.43 (d, J = 8.8 Hz, 1H), 7.23−7.51 (m, 5H + 1H), 7.50 (d, J = 9.2 Hz, 1H), 8.76 (s, 1H), 9.90 ppm (d, J = 6.4 Hz, 1H); ESI-MS obsd 598.4 $[M - H]^-$, calcd 598.24.

P-coum 3. Pd−C (10 mg) was added to the solution of coumarin-*O*-benzyloxy-phosphono-L-serin cyclohexyl **8** in choloroform/methanol (4 mL/8 mL). The mixture solution was stirred at room

(22) Matsumoto, S.; Yamaguchi, S.; Ueno, S.; Komatsu, H.; Ikeda, M.; Ishizuka, K.; Iko, Y.; Tabata, K. V.; Aoki, H.; Ito, S.; Noji, H.; Hamachi, I. *Chem.−Eur. J.* **2008**, *14*, 3977–3986.
(23) Li, J.-S.; Wang, H.; Cao, L.-W.; Zhang, H.-S. *Talanta* **2006**, *69*, 1190–1199.

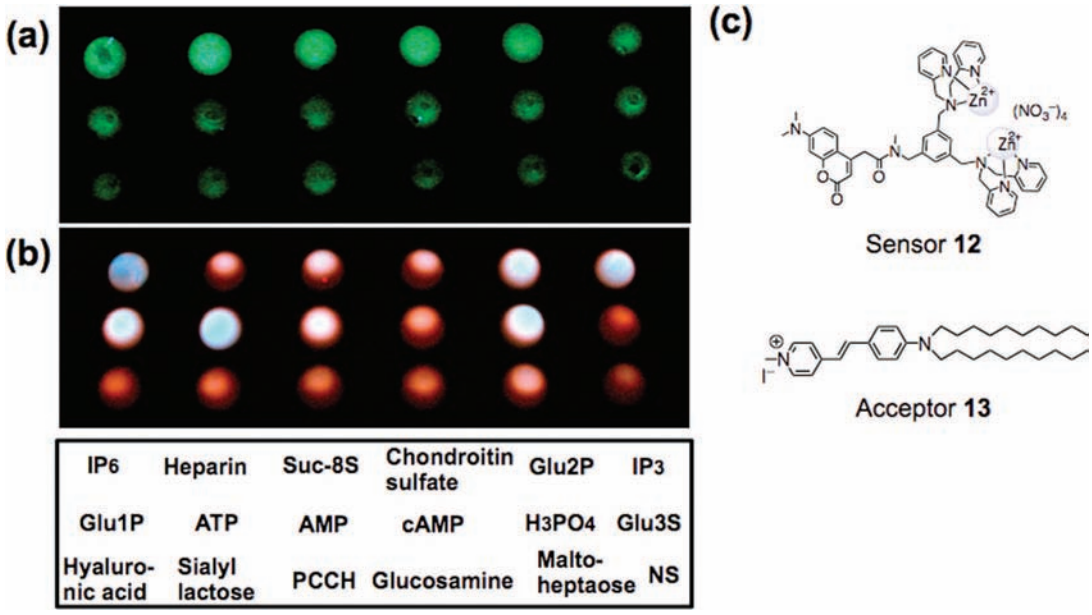
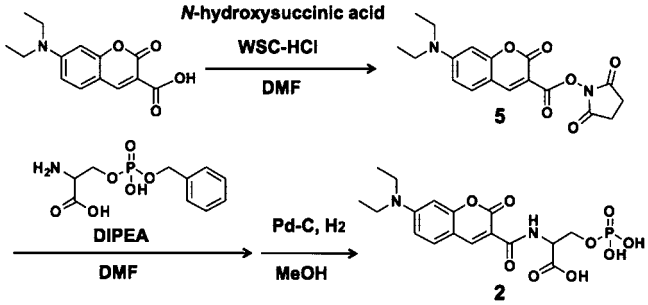


Figure 9. Photographs of (a) MCM−enzyme−supramolecular hydrogel hybrid sensory chip comprising hydrogel **1** containing BODIPY **4**, P-coum **3** encapsulated NH_2−MCM, and ACP and (b) phosphate anion-sensing chip of hydrogel **1** containing fluorescent receptor **12** and the FRET acceptor **13** for anionic species assay. The spotted positions of anions are shown at the bottom. Conditions: Spotted volume = 10 μL. (a) [**1**] = 0.090 wt % (200 mM acetate buffer, pH 5.0), [**4**] = 3.0 μM, [P-coum **3**@NH_2−MCM] = 5.0 mg mL^{-1} (50 μg), [anion] = 1.5 mM (15 nmol). (b) [**1**] = 0.10 wt % (50 mM HEPES buffer, pH 7.2, 100 mM NaCl), [**12**] = 50 μM, [**13**] = 30 μM, [anion] = 0.50 mM (5.0 nmol), RT. See Supporting Information for the structures of the anions. (c) Chemical structures of **12** and **13**.

Scheme 2. Synthesis of P-coum (**2**)



temperature under H_2 atmosphere. After 2 h, Pd−C was removed by filteration, and the solvent was removed under reduced pressure to afford compound **3** as a yellow solid (16 mg, 100%). ^{1}H NMR (400 MHz, DMSO-d_6, room temperature): δ 1.09 (t, J = 6.4 Hz, 6H), 1.13−1.67 (m, 10H), 2.44−2.45 (q, 4H (overlapped with DMSO)), 3.33−3.44 (m, 1H (overlapped with H_2O)), 3.92−3.99 (m, 2H), 4.59−4.63 (m, 1H), 6.58 (s, 1H), 6.75 (d, J = 9.6 Hz, 1H), 7.62 (d, J = 8.8 Hz 1H), 7.99 (d, J = 6.8 Hz, 1H), 8.61 (s, 1H), 9.09 ppm (d, J = 6.8 Hz, 1H); FAB-HRMS obsd 510.2007 $[M + H]^+$, calcd 510.2005 ($C_{23}H_{33}N_3O_8P$).

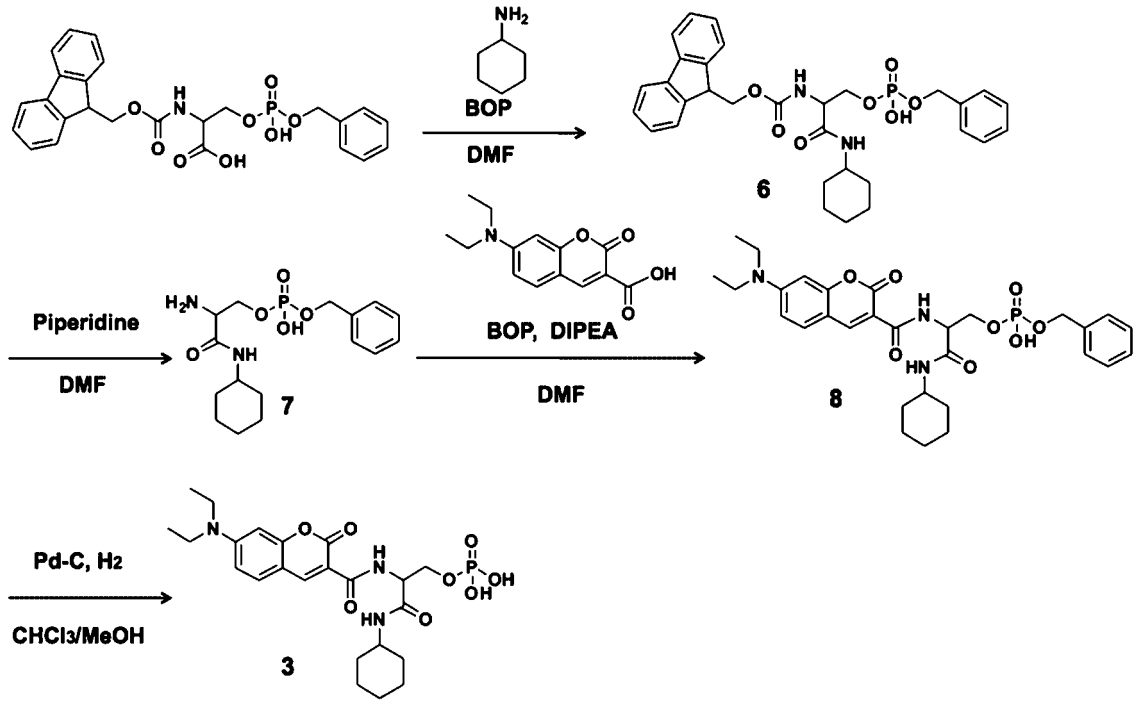
BODIPY-OSu (9). A solution of BODIPY−carboxylic acid (150 mg, 0.38 mmol), *N*-hydroxysuccinic imide (65 mg, 0.57 mmol), and water-soluble carbodiimide hydrochloride (WSC-HCl, 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimde hydrochloride, 108 mg, 0.57 mmol) in dry DMF (5 mL) was stirred at room temperature under Ar atmosphere. After 2.5 h, the solvent was removed under reduced pressure. The residue was dissolved in chloroform (100 mL), and the solution was washed with brine (100 mL). The organic layer was collected and dried over anhydrous Na_2SO_4 and filtered. The filtrate was concentrated to dryness, and the residue was purified by column chromatography (SiO_2, chloroform/methanol = 30:1), affording compound **9** as a red solid (136 mg, 53%). ^{1}H NMR (400 MHz, $CDCl_3$, room temperature): δ 1.41 (s, 6H), 2.55 (s, 6H), 2.89 (s, 4H), 5.05 (s, 2H), 5.98 (s, 2H), 7.08 (d, J = 8.8 Hz, 2H), 7.25 ppm (d, J = 8.8 Hz, 2H).

BODIPY Pentanoic Acid (10). A solution of BODIPY-OSu **9** (100 mg, 0.20 mmol) and 5-aminopentanoic acid (35 mg, 0.30 mmol) in DMF/methanol (3 mL/8 mL) was stirred at room temperature under Ar atmosphere. After 1 h, the solvent was removed under reduced pressure. The residue was dissolved in chloroform (100 mL), and the solution was washed with 5% aqueous citric acid (100 mL) and brine (100 mL). The organic layer was collected and dried over anhydrous Na_2SO_4 and filtered. The filtrate was concentrated to dryness, and the residue was purified by column chromatography (SiO_2, chloroform/methanol = 10:1), affording compound **10** as a red solid (89 mg, 90%). ^{1}H NMR (400 MHz, $CDCl_3$, room temperature): δ 1.41 (s, 6H), 1.56−1.71 (m, 2H + 2H), 2.42 (t, J = 6.8 Hz, 2H), 2.55 (s, 6H), 3.40 (q, J = 6.0 Hz, 2H), 4.55 (s, 2H), 5.98 (s, 2H), 6.72 (br s, 1H), 7.05 (d, J = 8.8 Hz, 2H), 7.23 ppm (d, J = 8.8 Hz, 2H); ESI-MS obsd 520.4 $[M + Na]^+$, calcd 520.23.

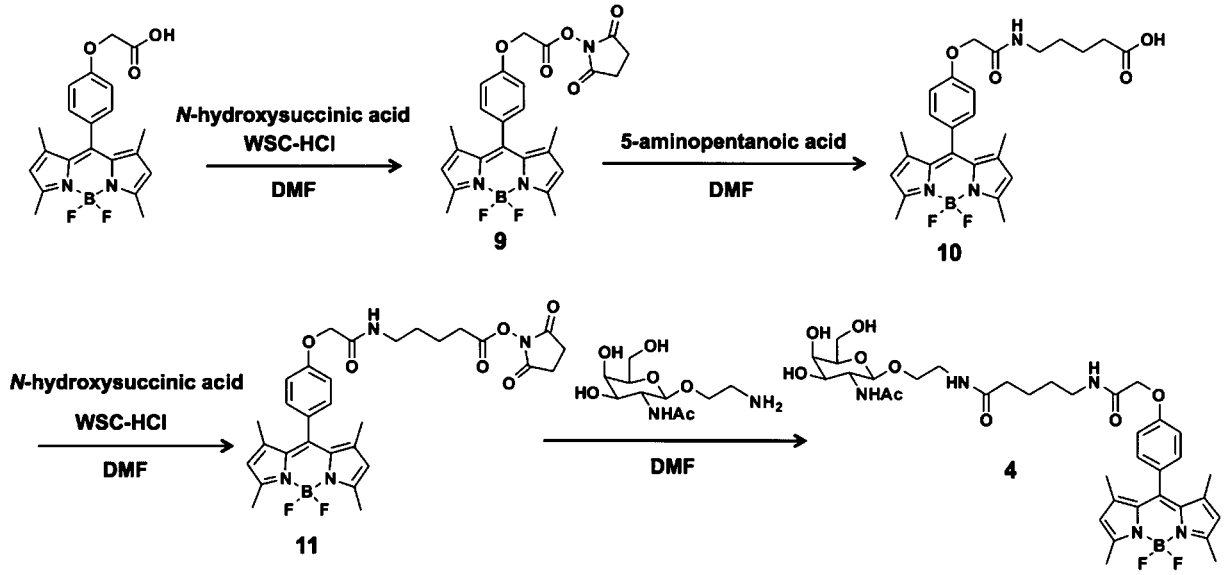
BODIPY Pentanoic Acid-OSu (11). A solution of BODIPY pentanoic acid **10** (44 mg, 0.088 mmol), *N*-hydroxysuccinic imide (15 mg, 0.13 mmol), and WSC-HCl (25 mg, 0.13 mmol) in dry DMF (5 mL) was stirred at room temperature under Ar atmosphere. After 5 h, the solvent was removed under reduced pressure. The residue was dissolved in chloroform (50 mL), and the solution was washed with brine (50 mL). The organic layer was collected and dried over anhydrous Na_2SO_4 and filtered. The filtrate was concentrated to dryness, and the residue was purified by column chromatography (SiO_2, chloroform/methanol = 30:1 to 20:1), affording compound **11** as a red solid (48 mg, 92%). ^{1}H NMR (400 MHz, $CDCl_3$, room temperature): δ 1.41 (s, 6H), 1.69−1.73 (m, 2H), 1.81−1.84 (m, 2H), 2.55 (s, 6H), 2.68 (t, J = 6.8 Hz, 2H), 2.84 (s, 4H), 3.42 (q, J = 6.4 Hz, 2H), 4.55 (s, 2H), 5.99 (s, 2H), 7.05 (d, J = 8.4 Hz, 2H), 7.23 ppm (d, J = 8.4 Hz, 2H).

GalNAc-BODIPY (4). A solution of BODIPY pentanoic acid−OSu **11** (15 mg, 0.025 mmol) and GalNAc−NH_2 (9.0 mg, 0.034 mmol) in dry DMF (2 mL) was stirred at room temperature under Ar atmosphere. After 2 h, the solvent was removed under reduced pressure. The residue was purified by column chromatography (SiO_2, chloroform/methanol = 10:1 to 7:1), affording compound **4** as a red solid (14 mg, 76%). ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$ = 7:1, room temperature): δ 1.31 (s, 6H), 1.45−1.52

Scheme 3. Synthesis of P-coum (**3**)



Scheme 4. Synthesis of BODIPY (**4**)



(m, 2H), 1.55−1.56 (m, 2H), 1.91 (s, 3H), 2.13 (t, J = 6.8 Hz, 2H), 2.43 (s, 6H), 3.22−3.26 (m, 2H + 2H (overlapped with methanol)) 3.36−3.54 (m, 2H + 2H), 3.56−3.60 (m, 1H + 1H), 3.63−3.70 (m, 1H + 1H), 4.25 (d, J = 8.0 Hz, 1H), 4.43 (s, 2H), 5.89 (s, 2H), 6.98 (d, J = 8.0 Hz, 2H), 7.12 ppm (d, J = 8.0 Hz, 2H); FAB-HRMS obsd 743.3529 $[M]^+$, calcd 743.3513 ($C_{36}H_{48}N_5O_9F_2B_1$).

Preparation of P-coum 2 Encapsulated NH_2−MCM. NH_2−MCM (1.0 mg) was suspended in P-coum **2** solution (316 μM, pH 5.0, 200 mM acetate, 100 μL), and the resultant susupension was vortexed overnight. The suspension was centrifugated (4000 rpm for 8 min), and supernatant was removed. The P-coum **2** encapsulated NH_2−MCM was washed with 200 mM acetate (pH 5.0, 500 μL) four times. All supernatants were collected, and unencapsulated P-coum **2** was determined by UV−vis spectra. P-coum **2** loaded in 1.0 mg of NH_2−MCM was determined to be 28 ± 0.3 nmol (81 ± 5%).

Preparation of P-coum 3 Encapsulated NH_2−MCM. NH_2−MCM (1.0 mg) was suspended in P-coum **3** solution (103 μM, pH 5.0, 200 mM acetate, 324 μL), and the resultant susupension was vortexed overnight. The suspension was centrifugated (4000 rpm for 8 min), and supernatant was removed. The P-coum **3** encapsulated NH_2−MCM was washed with 200 mM acetate (pH 5.0, 500 μL) four times. All supernatants were collected, and unencapsulated P-coum **3** was determined by UV−vis spectra. P-coum **3** loaded in 1.0 mg of NH_2−MCM was determined to be 17 ± 0.9 nmol (49 ± 1%).

Preparation of Supramolecular Hydrogel Containing Fluorescent Dye 2 Encapsulated NH_2−MCM. A suspension of gelator **1** (1.0 mg) in 200 mM acetate buffer (pH 5.0, 1.0 mL) or 50 mM Tris-HCl buffer (pH 5.0, 1.0 mL) was heated to form homogeneous solution. This hot solution was added to a suspension of P-coum **2** encapsulated NH_2−MCM in aqueous buffer. The

suspension was pipetted, and the resultant homogeneous suspension was dropped into hexadecane to form gel droplets (0.5−1.0 μL) for CLSM observations or spotted on a slide glass plate and incubated to complete gelation in a sealed box with high humidity at room temperature for 30 min for MCPD measurements.

Preparation of Supramolecular Hydrogel Containing Fluorescent Dye 3 Encapsulated NH_2−MCM and a FRET Acceptor (BODIPY 4). A BODIPY **4** solution in MeOH (2 mM, 2 or 3 μL) was added to a suspension of gelator **1** (1.0 mg) in 200 mM acetate buffer (pH 5.0, 1.0 mL) or 50 mM Tris-HCl buffer (pH 5.0, 1.0 mL), and the mixture was heated to form homogeneous solution. This hot solution was added to a suspension of P-coum **3** encapsulated NH_2−MCM in aqueous buffer. The suspension was pipetted, and the resultant homogeneous suspension was dropped into hexadecane to form gel droplets (0.5−1.0 μL) for CLSM observations or spotted on a slide glass plate and incubated to complete gelation in a sealed box with high humidity at room temperature for 30 min for MCPD measurements.

pH and Polyanion Responses of Fluorescent Dye Encapsulated NH_2−MCM−Enzyme−Supramolecular Hydrogel Hybrids. Hydrogel **1** (0.09 wt %) containing **2** or **3** encapsulated NH_2−MCM (9 μg μL^{-1}) on a slide glass plate was prepared according to the procedure described above. After the pH was adjusted from 5 to 10 and an anion species solution was added, alkaline phosphatase (AP, 1 μL, 6 m units μL^{-1}) and acid phosphatase (ACP, 1 μL, 6 m units μL^{-1}) were added to the hybrid hydrogel spots, respectively, and the slide glass plates were incubated in a sealed box with high humidity at room temperature for 60 min. The fluorescence spectra of the hybrid hydrogel spots were traced by MCPD at room temperature. For droplet experiments, a hydrogel droplet (0.09 wt %, 200 mM CHES buffer, pH 10) containing AP (0.55 m unit μL^{-1}) was fused with the hybrid hydrogel droplet and the process was visualized by CLSM observations.

Preparation of Polyanion Recognition Supramolecular Gel Chips. BODIPY **4** (3 μM) appended hydrogel **1** (0.09 wt %) containing NH_2−MCM (5 μg μL^{-1}) on a slide glass plate was prepared according to the procedure described above. Anion species solution (15 mM, 1 μL) was added to the hydrogel spots, and the slide glass was incubated in a sealed box with high humidity at room temperature for 60 min. Then, ACP (2 μL, 24 m units μL^{-1}) was added to the hydrogel spots, and the slide glass was incubated in a sealed box with high humidity at room temperature for 90 min. The fluorescence spectra of the hydrogel spots were traced by MCPD at room tempreture. The photographs of the resulting hydrogel chip were collected by using a digital camera under UV irradiation (λ_{ex} = 365 nm, using a handy lamp equipped with a cutoff filter (<300 nm and >400 nm)).

Preparation of Phosphate Anion Recognition Supramolecular Gel Chips. A styryl dye **13** (30 μM) appended hydrogel **1** (0.10 wt %) containing coumarin-appended phosphate receptor **12** (50 μM) on a slide glass plate was prepared according to the procedure reported previously.[15a] Anion species solution (5 mM, 1 μL) was added to the hydrogel spots, and the slide glass plate was incubated in a sealed box with high humidity at room temperature for 10 min. The photographs of the resulting hydrogel chip were collected by using a digital camera equipped with a cutoff filter (<420 nm) under UV irradiation (λ_{ex} = 365 nm, using a handy lamp).

CLSM Observations. The sample was spotted on a glass-bottom dish (Matsunami, noncoat, 0.15−0.18-mm glass bottom) and subjected to CLSM observation using an inverted confocal laser scanning microscope (Olympus FV1000-ASW) equipped with a 543- and 633-nm Helium Neon, 458- and 488-nm multi-Ar lasers (Coherent Inc.). A 100× NA = 1.40 oil objective or a 10× NA = 0.40 air objective was employed to obtain images.

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Supporting Information Available: Synthetic procedures for NH_2−MCM41 and P-coum **2**, Figures S1−S5, and complete author list for refs 2 and 6. This material is available free of charge via the Internet at http://pubs.acs.org.

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