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Chemically Reactive Supramolecular Hydrogel Coupled with a Signal Amplification System for Enhanced Analyte Sensitivity

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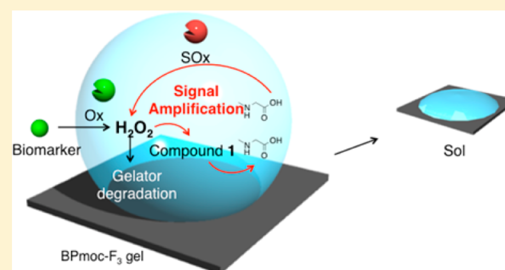
Supporting Information

ABSTRACT: Multicomponent supramolecular hydrogels are constructed for sensitive, naked-eye detection of small-molecule biomarkers. A dendritic self-immolative molecule and the corresponding enzyme as a signal amplification system were stably embedded in a hydrogen peroxide (H_2O_2)-responsive supramolecular hydrogel (**BPmoc-F$_3$**), together with other enzymes. The nanostructure and mechanical strength of the hybrid **BPmoc-F$_3$** gel were not substantially diminished by incorporation of these multiple components in the absence of target biomarkers, but could be destroyed by addition of the biomarker through the multiple enzymatic and chemical cascade reactions operating in combination within the gel matrix. The sensitivity to biomarkers such as H_2O_2, glucose, and uric acid, detected by gel−sol transition, was significantly enhanced by the signal amplification system. An array chip consisting of these multicomponent hydrogels enabled the detection of the level of hyperuricemia disease in human plasma samples.



INTRODUCTION

In the design of soft-materials, living cells present many attractive functions.[1] The presence of multiple components that are capable of functioning cooperatively and/or orthogonally in a single cell allows them to respond flexibly to environmental changes. Supramolecular hydrogels[2] exhibiting macroscopic responses to various stimuli are promising soft materials that have applications in biosensors,[3] controlled drug release,[4] and regenerative medicine.[5] Pioneering efforts aimed at mimicking sophisticated cellular functions have been initiated recently on combining multiple components within supramolecular hydrogels[6] and on the elaborate molecular design of the gelator itself. For example, van Esch and co-workers developed a drug delivery platform by orthogonally encapsulating micelles or enzyme-loaded liposomes into a supramolecular hydrogel.[7] This three-component gel enabled precise control of drug release rate by heating time. As a unique fluorescent sensor array, we constructed multicomponent hydrogels incorporating mesoporous silica (or layered clay), fluorescent dyes, and phosphatase.[8] More recently, we developed chemically reactive supramolecular hydrogels, in which several enzymes and a different type of gelator are embedded, to create unique logic-gated responses to biomarkers.[9] However, their sensitivity for detecting biomarkers in pathological conditions remained low in some cases, and functional components that work synergistically within supramolecular hydrogels are still very limited.

Recently, chemical approaches for construction of signal amplification system have been paid much attention.[10] Many of them rely on the analyte-induced activation of catalysts, where enzymes,[11] ribozymes,[12] metal nanoparticles,[13] and others[14] are used for signal amplification. The turnover of these catalysts leads to the enhanced conversion of chromogenic substrate. Another approach exploits self-immolative polymers[15] or dendrimers,[16] in which selective cleavage of the end groups triggers the sequential fragmentation to generate multiple reporters. More recently, Shabat and co-workers reported a unique signal amplification system called dendritic chain reaction.[17] It combines the dendritic self-immolative molecule and enzymes to facilitate autocatalytic cycles.

Inspired by such precedented examples on signal amplification system with chemosensors, we here sought to construct naked-eye detectable systems comprising of the chemically reactive supramolecular hydrogel and a signal amplification system (Figure 1). We demonstrated that incorporation of the signal amplification system into an H_2O_2-responsive **BPmoc-F$_3$** hydrogel effectively enhances its sensitivity to H_2O_2, glucose, and uric acid. In particular, a multicomponent hydrogel containing the synthetic amplifier/sarcosine oxidase (SOx)/urate oxidase (UOx) successfully created a user-friendly, naked eye detection sensor for the level of uric acid (gout) in human plasma.

RESULTS AND DISCUSSION

Molecular Design and Synthesis. The signal amplification system was constructed by the combination of amplifier **1** and SOx (Figure 1). Amplifier **1** was designed on the basis of a dendritic self-immolative molecule previously reported by Shabat et al.[17] Their original amplifier contained a phenylboronic acid and two choline moieties linked through

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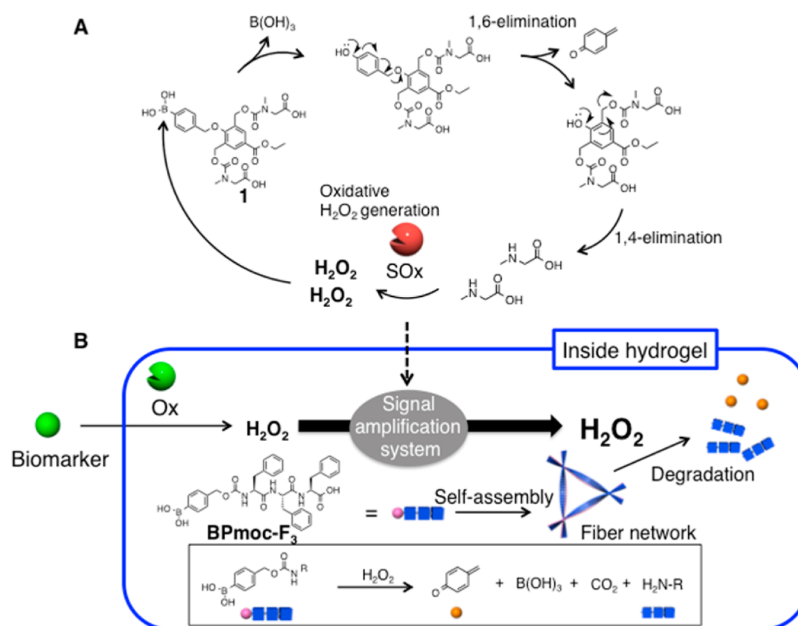

Figure 1. (A) Dendritic chain reaction of **1** and SOx. When H_2O_2 emerges, it attacks the boronic acid of the amplifier **1**, followed by 1,6-elimination and the subsequent 1,4-elimination reaction to facilitate the release of two sarcosines. The enzymatic oxidation of SOx produces H_2O_2 again to continue the reaction cycle. (B) Schematic representation of biomarker detection in supramolecular hydrogel (**BPmoc-F$_3$**) encapsulating signal amplification system (**1** + SOx).

carbonate bonds. However, a carbonate linkage is susceptible to hydrolysis, which may lead to spontaneous gel−sol transition in the absence of analytes (false positive). Therefore, we decided to replace it with the carbamate bond by using sarcosine instead of choline as the enzyme substrate during the amplification process. In the new amplifier **1**, the boronic acid moiety reacts with H_2O_2 to generate sarcosine by the spontaneous 1,6-elimination, 1,4-elimination, and decarboxylation reaction. The two released sarcosine molecules are oxidized by SOx, generating two H_2O_2 molecules as byproducts. This chain reaction amplifies H_2O_2 production (Figure 1A). By combining **1** and SOx as a signal amplification system into a hydrogel consisting of **BPmoc-F$_3$**, a H_2O_2-responsive hydrogelator, it was expected that the amplified H_2O_2 would chemically destroy **BPmoc-F$_3$** to efficiently induce the gel−sol transition (Figure 1B). Moreover, sensitive naked-eye sensing of various analytes could be carried out through the gel−sol transition if the analytes can produce H_2O_2 through the corresponding enzymatic reactions.

Impact of Amplifier 1 and Enzymes on the Gel State of BPmoc-F$_3$. Encapsulation of multiple components, other than the gelator, could cause disruption of the hydrogel state if any unfavorably interact with the fibrous nanostructures of **BPmoc-F$_3$**. We therefore investigated the properties of a **BPmoc-F$_3$** hydrogel encapsulating oxidases and amplifier **1** prior to examining the amplified analyte sensing. We indeed found that the gel did not form in the case of incorporating 10 equiv of amplifier **1**, while the gel state was retained with 5 or lower equiv of **1** relative to the **BPmoc-F$_3$** hydrogelator (Figure S1). From circular dichroism (CD) spectral analysis, a negative Cotton peak at 225 nm indicated formation of the β-sheet-like structure (Figure 2A),[18] which did not substantially change after addition of amplifier **1**. The nanostructure inside a **BPmoc-F$_3$** gel stained with a fluorescent dye (G-Coum)[8b] was observed by confocal laser scanning microscopy (CLSM). As shown in Figure 2B, thin and dense fibers were observed, even

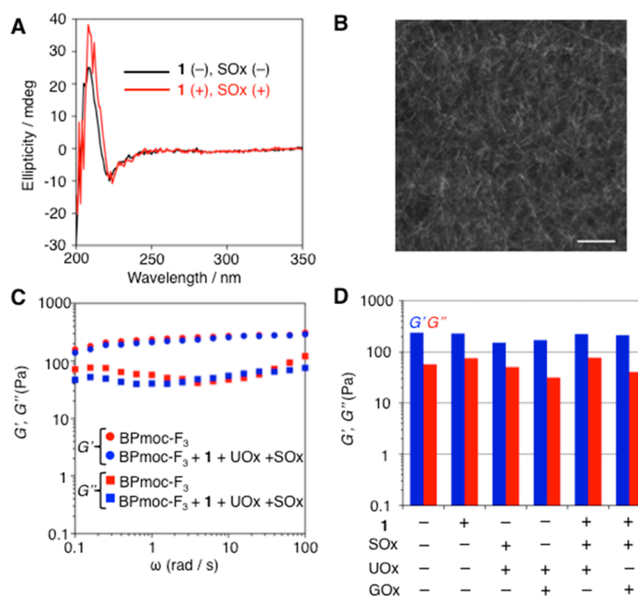

Figure 2. Properties of **BPmoc-F$_3$**. (A) CD spectra of **BPmoc-F$_3$** (black line) and **1** + SOx⊂**BPmoc-F$_3$** hybrid gel (red line). Conditions: [**BPmoc-F$_3$**] = 0.075 wt % (= 1.2 mM), 50 mM phosphate buffer (pH = 6.0, CGC: 0.05 wt %) [**1**] = 1.2 mM, [SOx] = 2.9 μM, 25 °C. (B) CLSM image of **1**⊂**BPmoc-F$_3$** gel stained with G-Coum. Conditions: [**BPmoc-F$_3$**] = 0.2 wt % (= 3.2 mM) in 200 mM MES (pH = 7.0, CGC: 0.05 wt %), [**1**] = 3.2 mM, Scale bar 5 μm. (C) Frequency sweep (1% strain amplitude) rheological properties of **BPmoc-F$_3$** gel and **1** + oxidases⊂**BPmoc-F$_3$** hybrid gel. (D) Rheological properties of **BPmoc-F$_3$** hybrid gels at an angular frequency of 1 rad/s. Conditions: [**BPmoc-F$_3$**] = 0.1 wt % (= 1.6 mM), 200 mM MES (pH = 7.0), [**1**] = 1.6 mM, [SOx] = 2.9 μM, [UOx] = 6.7 μM, 25 °C.

when encapsulating **1** (**1**⊂**BPmoc-F$_3$**, Figures 2B and S2C) or oxidases (such as SOx, GOx and UOx) in **BPmoc-F$_3$** gel (oxidases⊂**BPmoc-F$_3$**, Figure S2D), similar to the **BPmoc-F$_3$**

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gel alone (Figure S2B). Frequency sweep rheological experiments showed that G' (150−300 Pa) was larger than G'' (50−80 Pa), indicating that the **BPmoc-F$_3$** gel had the viscoelastic properties typical of a hydrogel consisting of fibrous networks (Figure 2C).[19] Importantly, no noticeable changes in these two values (G' and G'') were detected upon addition of **1** and one or two oxidases (Figure 2C and D). From these results, it was confirmed that the encapsulation of **1** and enzymes within the gel matrix did not diminish the fibrous nanostructure and mechanical properties of the **BPmoc-F$_3$** hydrogel. That is, the **BPmoc-F$_3$** gel was robust enough to maintain the gel state even when it contained the three additional components amplifier **1**, SOx, and glucose oxidase (GOx) or UOx.

Improvement of BPmoc-F$_3$ gel H_2O_2 Response by Signal Amplification. To test that the amplification system comprising **1** and SOx could efficiently induce the macroscopic gel−sol transition of **BPmoc-F$_3$**, we initially evaluated its sensitivity to H_2O_2, a biomarker for inflammation[20] and a simple substrate that triggers the dendritic chain reaction. As shown in Figure 3A, **1** + SOx⊂**BPmoc-F$_3$** exhibited the gel−sol transition when more than 0.16 mM of H_2O_2 was added. In contrast, the **BPmoc-F$_3$** gel alone needed a higher concentration of H_2O_2 to induce collapse (0.80 mM in Figure 3B). It is clear that the gel−sol transition occurred in the threshold mode in response to H_2O_2 concentration and the threshold value was decreased 5-fold by the signal amplification system. However, SOx⊂**BPmoc-F$_3$** (lacking amplifier **1**) and **1**⊂**BPmoc-F$_3$** (in the absence of SOx) did not show the gel−sol transition after addition of 0.32 mM H_2O_2 (Figure S3). HPLC analysis during the reaction confirmed that the (mole ratio) amount of decomposed **BPmoc-F$_3$** always exceeded that of added H_2O_2 in the **1** + SOx⊂**BPmoc-F$_3$** hybrid gel (Figure 3C), whereas the decomposition took place almost proportionally (that is 1:1 decomposed gelator:added H_2O_2) without the amplification system (Figure 3D). The HPLC data also revealed that the macroscopic gel-to-sol transition was induced when the residual gelator concentration fell below the critical gel concentration (CGC, 0.05 wt %), which caused the threshold type of response, one of the unique features of chemically reactive hydrogels.[9,21] These results strongly suggest that a small amount of H_2O_2 reacted with signal amplifier **1** to trigger the dendritic chain reaction, which resulted in H_2O_2 amplification, followed by the facilitated gel−sol transition. In addition, incorporation of **1** and SOx was proven to function as an excellent signal amplification system in the **BPmoc-F$_3$** gel matrix.[22]

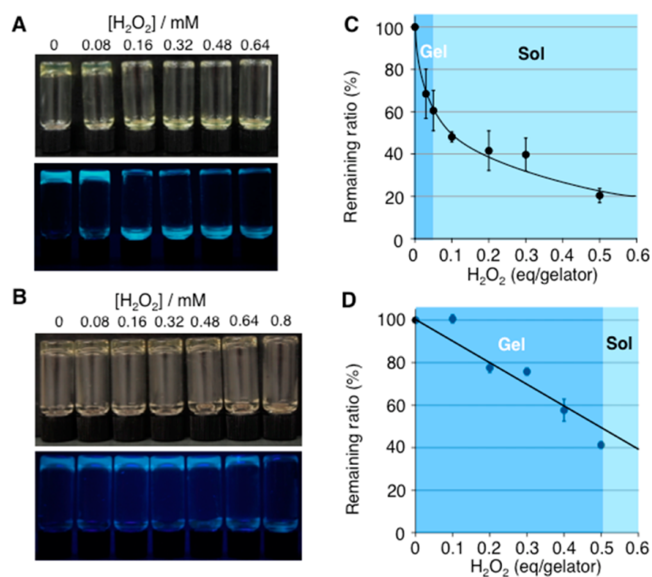

Figure 3. (A, B) Photographs of **BPmoc-F$_3$** gels after the addition of various amounts of H_2O_2. Fluorescence dye (7-dimethylaminocoumarin 3-carboxylic acid, DEAC) was added for clearly visualizing the gel−sol transition. (C, D) Remaining ratio of **BPmoc-F$_3$** after the addition of various amount of H_2O_2. The experiments were performed in triplicate to obtain mean and standard deviation values (shown as error bars). The lines are to guide the eye. (A, C) **BPmoc-F$_3$** gel containing **1** (1.6 mM) and SOx (2.9 μM). (B, D) **BPmoc-F$_3$** gel. Conditions: [**BPmoc-F$_3$**] = 0.10 wt % (1.6 mM), 100 mM MES (pH 7.0, CGC: 0.05 wt %), 37 °C, 12 h.

Enhanced Biomarker Sensitivity of the Multicomponent BPmoc-F$_3$ Gel. With an effective amplification system (**1** + SOx) in hand, we then expected that introduction of a second oxidase to this **BPmoc-F$_3$** hybrid gel could expand the range of analytes that could be sensed by the gel−sol transition. That is, given a small amount of H_2O_2 generated by an enzymatic reaction of a second encapsulated oxidase with its corresponding substrate (analyte), the amplification system would be triggered to increase the H_2O_2 concentration (Figure 1). Consequently, **BPmoc-F$_3$** decomposition would occur to cause the gel−sol transition. To provide an example for proof of principle, GOx was immobilized as the second oxidase in a **1** + SOx⊂**BPmoc-F$_3$** hybrid gel. In this multicomponent hydrogel, glucose, a biomarker for diabetes,[23] was expected to be sensed as the analyte. Figure 4A showed that the gel−sol transition occurred in the **1** + SOx + GOx⊂**BPmoc-F$_3$** hybrid gel when

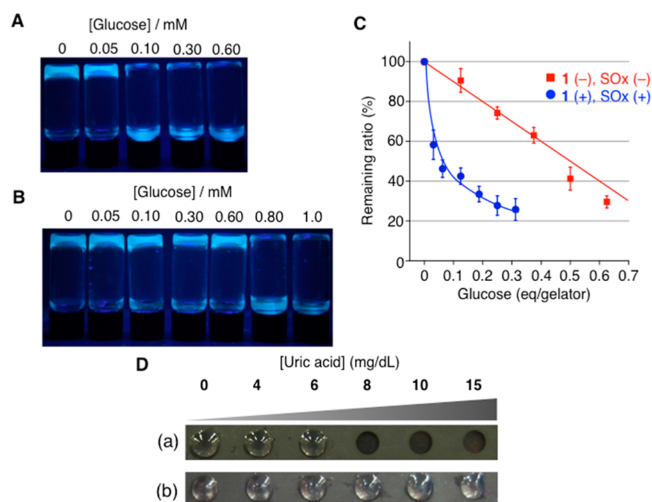

Figure 4. (A, B) Photographs of **BPmoc-F$_3$** gels after the addition of various amount of Glucose. DEAC was added for clearly visualizing the gel−sol transition. (A) **1** + SOx + GOx⊂**BPmoc-F$_3$** hybrid gel. (B) GOx⊂**BPmoc-F$_3$** hybrid gel. (C) Plots of remained **BPmoc-F$_3$** (%) after the addition of various amount of glucose. The experiments were performed in triplicate to obtain mean and standard deviation values (shown as error bars). The lines are to guide the eye. Conditions: [**BPmoc-F$_3$**] = 0.10 wt % (1.6 mM), 100 mM MES (pH 7.0), [SOx] = 2.9 μM, [GOx] = 3.3 μM, 37 °C, 12 h. (D) Photograph of gel array on a flat glass slides after the response to blood plasma that contained various amounts of uric acid: (a) **1** + SOx + UOx⊂**BPmoc-F$_3$** hybrid gel and (b) UOx⊂**BPmoc-F$_3$** hybrid gel. Conditions: [**BPmoc-F$_3$**] = 0.075 wt % (1.2 mM), 100 mM MES (pH 7.0), [**1**] = 1.6 mM, [SOx] = 2.9 μM, [UOx] = 8.3 μM, V(gel):V(blood plasma) = 5:1, 37 °C, 3 h.

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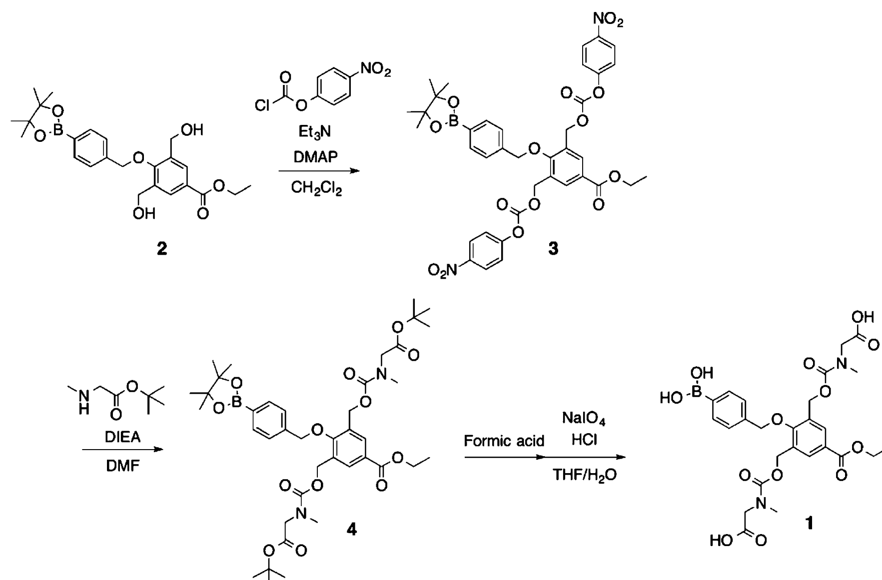
Scheme 1. Synthesis of Amplifier 1



0.10 mM of glucose was added, whereas higher than 0.80 mM of glucose was required for the gel−sol transition of GOx⊂**BPmoc-F**$_3$ (without the amplification system, Figure 4B). The glucose sensitivity was thus apparently improved 8-fold using the signal amplification system. The HPLC analysis was consistent with this enhanced sensitivity and showed that the residual **BPmoc-F**$_3$ was lower than the CGC (Figure 4C) in cases where gel collapse was induced. These HPLC data indicated that quantitative detection of biomarkers is also possible by determining the amount of the remained gelator as well as the qualitative naked-eye detection.

In these new multicomponent hydrogels, secondary oxidases, such as GOx, control analyte selectivity, while **1** + SOx amplifies the signal. Thus, the second oxidase immobilized in **1** + SOx⊂**BPmoc-F**$_3$ can be replaced with various oxidases, depending on the desired target analyte. We sought to construct a hybrid gel sensor with high sensitivity for uric acid, an important biomarker of gout,[24] by encapsulating UOx in **1** + SOx⊂**BPmoc-F**$_3$. Upon addition of a uric acid concentration that corresponds to the critical plasma concentration in hyperuricemic patients (0.40 mM), **1** + SOx + UOx⊂**BPmoc-F**$_3$ exhibited the typical gel−sol transition (Figure S4A), while such transition was not observed in case of uric acid addition lower than the standard value in plasma (0.30 mM). UOx⊂**BPmoc-F**$_3$ (not containing **1** + SOx), by contrast, required 1.0 mM of uric acid to induce gel collapse (Figure S4B). HPLC analysis again showed that **BPmoc-F**$_3$ was decomposed to a concentration lower than the CGC value (Figure S4C), whereas excess degradation did not occur in the case of hybrid gels lacking **1**, UOx, or SOx (Figures S5 and S6).

Finally, to evaluate the practical utility of the new system, an array-based assay for uric acid in human plasma was performed. Small amounts of human plasma containing various concentrations of uric acid were added to **1** + UOx + SOx⊂**BPmoc-F**$_3$ hybrid gel spots prepared on a glass plate. After 3 h, the glass plate was gently washed with water to remove the sol spots, but not the gel spots. As shown in Figure 4D-a, the gel spots subjected to addition of more than 8 mg/dL of uric acid (corresponding to the hyperuricemic condition) were washed away. On the contrary, gel spots lacking **1** and SOx (comprising simply UOx⊂**BPmoc-F**$_3$) remained on the plate even after the addition of uric acid and the subsequent washing operation (Figure 4D-b). These results clearly demonstrate that the multicomponent hybrid gel can work effectively even in the presence of plasma containing highly complex constituents and that the signal amplification system indeed enhanced the sensitivity to levels close to the practical values required to conveniently monitor disease conditions.

■ CONCLUSION

In summary, we constructed a signal-amplifiable supramolecular hydrogel by encapsulating the self-immolative dendritic molecule **1** together with SOx. The pair **1** + SOx did not disrupt the supramolecular nanofibers of **BPmoc-F**$_3$ but worked in combination with the chemically reactive hydrogel, as a universal signal amplification system involving H_2O_2. This multiple component strategy allows us to flexibly construct hydrogel-based sensors with enhanced sensitivity toward biomarkers. The biomarker selectivity is tunable in a multi-component hydrogel through selection of a secondary oxidase that can trigger the signal amplification reactions through generation of H_2O_2. Of particular note, this amplification system can operate even with blood plasma samples containing highly complex constituents. This new simple, naked-eye detectable system holds promise for clinical diagnoses, especially in resource-limited environments.[25]

■ EXPERIMENTAL SECTION

General Materials and Methods. Unless stated otherwise, all commercial reagents were used as received. Glucose oxidase (from *Aspergillus niger*, SIGMA), sarcosine oxidase (from microorganism, TOYOBO), choline oxidase (from *Alcaligenes sp.*, TOYOBO), urate oxidase (from *Bacillus sp.*, TOYOBO) were used as received. Thin layer chromatography (TLC) was performed on silica gel 60F$_{254}$ (Merck). Column chromatography was performed on silica gel 60N (Kanto, 40−50 mm). Reverse phase HPLC (RP-HPLC) was conducted with a Hitachi Lachrom instrument equipped with YMC-pack Triart columns (250 × 30 mm i.d.) for purification or YMC-pack Triart columns (250 × 4.6 mm i.d.) for analysis. ^{1}H NMR spectra were obtained on a Varian Mercury 400 spectrometer with tetramethylsilane (TMS) or residual nondeuterated solvents as the internal references.

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Multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. ESI mass spectra were recorded using a Thermo Scientific Exactive mass spectrometer. The CD spectra were measured using a Jasco J-720WI spectropolarimeter.

Synthesis. Compound **1** was prepared as shown in Scheme 1.

*Synthesis of **3**.* To a stirred dry dichloromethane (CH_2Cl_2, 4 mL) solution of **2**[17b] (100 mg, 0.23 mmol), *N*,*N*-dimethyl-4-aminopyridine (DMAP, 2.2 mg, 18 μmol) and triethylamine (Et_3N, 95 μL, 0.69 mmol) were added 4-nitorophenyl chlorofomate (137 mg, 0.69 mmol). The mixture was stirred at room temperature (rt) for 3 h. The solvent was evaporated, and to the residue was added diethyl ether (30 mL). The appeared solid was removed by filtration. The residue was purified by column chromatography (silica, $CHCl_3$: AcOEt = 50:1) to give **3** (94 mg, 54%) as colorless oil. ^{1}H NMR ($CDCl_3$, 400 MHz, rt): δ = 1.36 (s, 12H), 1.42 (t, J = 7.2 Hz, 3H), 4.42 (q, J = 7.2 Hz, 2H), 5.13 (s, 2H), 5.37 (s, 4H), 7.29−7.33 (m, 4H), 7.46 (d, J = 8.0 Hz, 2H), 7.85 (d, J = 8.0 Hz, 2H), 8.23−8.28 ppm (m, 6H).

*Synthesis of **4**.* To a stirred dry *N*,*N*-dimethylformamide (DMF, 4 mL) solution of **3** (94 mg, 0.12 mmol) and diisopropylethylamine (DIEA, 150 μL, 0.78 mmol) was added sarcosine hydrochloride (55 mg, 0.30 mmol). The mixture was stirred at rt for 3h. The solvent was evaporated. The residue was diluted with CH_2Cl_2 (50 mL) and was washed with sat. $NaHCO_3$ aq. (40 mL × 5), H_2O (30 mL × 3), and brine (30 mL × 2). The organic layer was dried over $MgSO_4$. The residue was purified by column chromatography (silica, $CHCl_3$:AcOEt = 20:1) to give **4** (56 mg, 59%) as colorless oil. ^{1}H NMR ($CDCl_3$, 400 MHz, rt): δ = 1.35−1.45 (m, 33H), 2.95−2.97 (m, J = 7.2 Hz, 6H), 3.88 (d, 30.4 Hz, 4 H), 4.37 (q, J = 7.2 Hz, 2H), 4.97−5.03 (m, J = 12.6 Hz, 2H), 5.19−5.24 (m, 4H), 7.45 (d, J = 8.0 Hz, 2H), 7.83 (d, J = 8.0 Hz, 2H), 8.07 ppm (dd, J = 7.2, 23.6 Hz, 2H).

*Synthesis of **1**.* Compound **4** (106 mg, 0.14 mmol) was dissolved in formic acid (8 mL). The mixture was stirred at rt for 5 h. The solvent was evaporated. The residue was dissolved in THF:H_2O = 1:4 (15 mL). To this mixture was added $NaIO_4$ (106 mg, 0.50 mmol). The mixture was stirred at rt for 5 h. The solvent was evaporated. The residue was purified by RP-HPLC (silica, ACN (0.1% TFA): H_2O (0.1% TFA) = 20:80 to 50:50) to give **1** (49 mg, 59%) as a white solid. ^{1}H NMR (CD_3CN, 400 MHz, rt): δ = 1.39 (t, J = 7.2 Hz, 3H), 2.93−2.96 (d, J = 10.4 Hz, 6H), 3.97 (s, 4H), 4.37 (q, J = 7.2 Hz, 2H), 4.99−5.07 (m, 2H), 5.18−5.24 (m, 4H), 7.50 (d, J = 8.0 Hz, 1H), 7.83 (d, J = 8.0 Hz, 2H), 8.05−8.10 ppm (dd, J = 2.0, 7.2 Hz, 2H).

■ ASSOCIATED CONTENT

Supporting Information

Figures and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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