ELECTRONIC SUPPLEMENTARY INFORMATION

Design of Single-Step Immunoassay Principle Based on the Combination of Enzyme-Labeled Antibody Release Coating and Hydrogel Copolymerized with Fluorescent Enzyme Substrate

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EXPERIMENTAL METHODS

Reagents and materials. Precleaned glass microscope slides (76 × 26 mm, 0.8–1.0 mm thick) were purchased from Matsunami Glass Industry (Osaka, Japan). Poly[dimethylsiloxane-co-methyl(3-hydroxylpropyl)siloxane]-*graft*-poly(ethylene glycol) methyl ether (PDMS-PEG), ALP-labeled goat anti-human IgG, human IgG, rabbit IgG, and goat IgG were purchased from Sigma-Aldrich (Milwaukee, WI, USA). 3-(Trimethoxysilyl)propyl methacrylate and fluorescein were purchased from Tokyo Chemical Industry (Tokyo, Japan). Polyethylene glycol (PEG; M_n = 20,000) and trehalose were purchased from Wako Pure Chemical Industries (Osaka, Japan). SILPOT 184 and SILPOT 184 CAT were purchased from Dow Corning Toray Co., Ltd. (Tokyo, Japan). 1,3-Dihydroxybenzene, 3-ketogulutaric acid, 4-dimethylaminopyridine (DMAP), allylamine, diethyl phosphoryl chloride, trimethylsilyl bromide (TMSBr), and 2-hydroxy-2-methylpropiophenone (HOMPP) were purchased from Tokyo Chemical Industry (Tokyo, Japan). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and triethylamine (TEA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Alkaline phosphatase (ALP), 4-methylumbelliferone (MUF), and 30% (w/v) acrylamide (acrylamide/bis-acrylamide, 37.5:1 (w/w)) were purchased from Sigma-Aldrich (Milwaukee, WI, USA).

*Synthesis of 7-hydroxycoumarin-4-acetic acid (**1**).* 1,3-Dihydroxybenzene (9.0 g, 81 mmol) was dissolved in 70% sulfuric acid (81 mL) at 0°C, and then, 3-ketoglutaric acid (11.9 g, 81 mmol) was added. The mixture was allowed to warm up to room temperature and stirred for a further 4 h. The resulting solution was poured onto crushed ice. The white precipitate was collected by filtration, washed with water and then ethyl acetate, and dried overnight under reduced pressure to yield **1** as a white solid (12.2 g, 68%). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.50 (d, J = 8.9 Hz, 1H), 6.72 (dd, J = 7.6, 2.0 Hz, 1H), 6.50 (d, J = 2.1 Hz, 1H), 6.0 (s, 1H), 3.79 (s, 2H)

*Synthesis of 2-(7-hydroxycoumarin-4-acetylamino)-propylene (CMH) (**2**).* **1** (11.7 g, 57.6 mmol) was dissolved in DMF (40 mL) at room temperature, and then, DMAP (8.0 g, 65.0 mmol), EDC (11.4 g, 59.4 mmol), and allylamine (3.7 g, 65.0 mmol) were added and the mixture stirred for 12 h. After the solvent was removed under reduced pressure, the residue was washed with ethyl acetate, 1 M hydrochloric acid, and finally pure water. The organic layer was dried over anhydrous sodium sulfate and concentrated under reduced pressure, yielding yellow oil that was further purified by column chromatography (silica gel, chloroform/methanol, 12:1) to give **2** as a white solid (3.3 g, 24 %). ^{1}H-NMR (400 MHz, Methanol-d_4) δ 7.61 (d, J = 9.2 Hz, 1H), 6.79 (d, J = 10.8 Hz, 1H), 6.70 (s, 1H), 6.18 (s, 1H), 5.81 (m, J = 16.8 Hz, 1H), 5.15, 5.09 (dd, J = 16.8,

12.0 Hz, 2H,), 3.80 (d, *J* = 5.6, 2H), 3.73 (s, 2H)

*Synthesis of [2-(7-diethoxyphosphoryloxy) coumarin-4-acetylamino]- propylene (**3**).* **2** (1.01 g, 3.90 mmol) was dispersed in DCM (20 mL), and TEA was added until **2** dissolved at room temperature. Diethyl phosphoryl chloride (3.36 g, 19.0 mmol) was added, and the mixture was stirred for 2 h. After the solvent was removed under reduced pressure, the residue was washed with ethyl acetate, 1 M hydrochloric acid, and then pure water. The organic layer was dried over anhydrous sodium sulfate and concentrated under reduced pressure to yellow oil that was further purified by column chromatography (silica gel, chloroform/methanol, 20:1) to give **3** as a yellow liquid (1.03 g, 66 %). ^{1}H-NMR(400 MHz, Chloroform-d) δ 7.65 (d, *J* = 8.4 Hz, 1H), 7.12 (s,1H), 7.10 (s, 1H), 6.80 (t, 1H), 6.31 (d, *J* = 15.2 Hz, 1H), 5.73 (m,1H), 5.06 (dd, *J* = 1.6 Hz, 1.6 Hz, 2H), 4.20 (q, 4H), 3.81 (d, *J* = 6 Hz, 2H), 3.67 (s, 2H), 1.31 (t, 6H)

*Synthesis of [2-(7-dihydroxy phosphoryloxy) coumarin-4-acetylamino]- propylene (CMP) (**4**).* TMSBr (18.6 g, 121 mmol) was added in a solution of **3** (0.48 g, 1.21 mmol) in DCM (10 mL), and the mixture was stirred at room temperature under an argon atmosphere. The resulting solid was collected by filtration, washed with diethyl ether, and dried under reduced pressure, yielding **4** as a white powder (0.33 g, yield 80%). ^{1}H-NMR(400 MHz, Deuterium oxide) δ 7.60 (d, 1H, *J* = 8.4 Hz), 7.14 (s, 1H),

7.11 (d, J = 1.6 Hz, 1H), 5.72 (m,1H), 5.00 (dd, J = 6.4 Hz, 2 Hz, 2H), 3.81 (s, 2H), 3.71 (d, J = 5.2 Hz, 1H); MS (FAB): *m/z* 340 $[M+1]^+$

Characterization of spectral properties. Fluorescence measurements were performed using a spectrofluorometer (excitation wavelength: 369 nm, emission wavelength: 460 nm).

Characterization of enzyme reaction and copolymerization sites of CMP monomer. To investigate the enzyme reaction site, we used a spectrofluorometer to monitor spectral changes in fluorescence in buffer (50 mM Tris-HCl, pH 8.4) containing CMP (10 μM) and lower concentrations of ALP (0, 0.5, 1.0, 5.0, 7.5 mU/mL).

To investigate the effect of the copolymerization site, we prepared hydrogels containing CMH and a similar compound without the copolymerization site (MUF). Prepolymer solutions were prepared as follows: First, CMH (1 mM) and MUF (1 mM) in Tris-HCl (pH 8.0)/methanol (9:1 v/v) were separately prepared. Then, 100 μL of CMH or MUF solution was mixed with 40 μL of 30% (w/v) acrylamide, 58 μL of Tris-HCl (pH 8.0), and 2 μL of HOMPP. Blank prepolymer solution containing 40 μL of 30% (w/v) acrylamide, 158 μL of Tris-HCl (pH 8.0), and 2 μL of HOMPP was also prepared. The prepolymer solutions prepared (100 μL) were added to a microtube (ID, 0.5 cm; length, 5 cm), and UV irradiated (252 W, 2 min). This was followed by 3

washes with 400 μL of Tris-HCl (pH 8.0), and then 300 μL of Tris-HCl (pH 8.0) was introduced into the microtube and the fluorescence image obtained.

Fabrication of PDMS channel array. PDMS channel arrays were fabricated by a simple molding procedure using glass molds. PDMS prepolymer (SILPOT 184/SILPOT CAT at 10:1 (w/w)) was introduced into a glass mold. After the surface was covered with a thick PDMS plate and heated for 2 h, the PDMS channel array was peeled off the glass mold. In this study, 2 PDMS channel arrays were fabricated, one 500 μm wide × 200 μm deep, and the other, 700 μm wide × 500 μm deep, using the corresponding glass molds.

Fabrication of gel microchip. Glass slides were washed with 1 M sodium hydroxide (30 min), flushed with pure water, then with acetone, and dried. The slides were silanized by immersion for 5 h in 3-(trimethoxysilyl)propyl methacrylate/0.1 M hydrochloric acid (15:4 (v/v)) solution, washed with methanol, then with pure water, and dried. Coated slides were combined with a PDMS channel array, and prepolymer solution, containing 100 μL of 10 μM CMP monomer solution, 40 μL of 30% (w/v) acrylamide, 58 μL of Tris-HCl (pH 8.0), and 2 μL of HOMPP, was introduced into the microchannel and UV irradiated (252 W) for 2 min. Finally, the PDMS channel array was peeled off the slide, and the gel-microchip was washed with pure water, and then

Tris-HCl buffer (pH 8.0).

Fabrication of single-step microfluidic immunoassay device. The surface of the 700 μm wide × 500 μm deep PDMS channel array was hydrophilized by O_2 plasma generated in a low-pressure plasma system (100 W) for 2 min. A solution containing 7 mg/mL PEG, 1.9 μg/mL ALP-labeled anti-human IgG, 5 mg/mL trehalose, and 0.1% (v/v) PDMS-PEG was then introduced into the PDMS channels and dried under reduced pressure for 2 h. Thereafter, the PDMS channel array modified with a soluble coating was combined with a gel-microchip to fabricate the single-step immunoassay device.

Characterization of hydrogel-immobilizing fluorescent enzyme substrate. Fifty microliters of Tris-HCl buffer (pH 8.0) containing 190 ng/mL ALP-labeled anti-human IgG and 0 or 46 μg/mL human IgG was applied to the gel microchip, and the fluorescence intensity was measured for 60 min using a fluorescence microscope. For selectivity evaluation, 50 μL of Tris-HCl buffer (pH 8.0) containing 190 ng/mL ALP-labeled anti-human IgG and 250 ng/mL or 50 μg/mL of human, rabbit, or goat IgG, was applied to the gel microchip, and the fluorescence intensity was measured for 60 min as described above. In order to compensate for chip-to-chip variations in the amount of immobilized substrate, we plotted the ratio of the fluorescence intensity change ($F-F_0$) to the initial background fluorescence (F_0).

Single-step immunoassay. Tris-HCl buffer (pH 8.0) containing different concentrations of human IgG was introduced into the PDMS channel of the device, and fluorescence intensity was measured using a fluorescence microscope.

Electronic Supplementary Material (ESI) for Lab on a Chip

SUPPLEMENTAL RESULTS AND DISCUSSION

Characterization of CMP monomer. CMP monomer was designed to react with ALP enzyme, a common label for antibodies used in immunoassays, and to covalently attach to the hydrogel network. The synthesis of CMP was divided into 3 major steps (Fig. S1): (a) formation of the fluorescent coumarin backbone structure through the reaction of 1,3-dihydroxybenzene and ketoglutaric acid; (b) alkene functionalization of the carboxylic acid moiety using EDC chemistry in the presence of allylamine, leading to the formation of the CMH molecule; and (c) phosphorylation of the hydroxyl moiety using diethylphosphoryl chloride, followed by cleavage of the ethoxy group of the phosphoryl moiety by treatment with trimethylsilyl bromide.

The newly synthesized CMP was characterized by monitoring its reaction with ALP enzyme and its ability to copolymerize with the acrylamide monomer to form a hydrogel polymer. For monitoring the reaction with ALP, 10 μM CMP was allowed to react with various concentrations of ALP, and the formation of fluorescent CMH molecules was monitored at excitation and emission wavelengths of 369 nm and 460 nm, respectively. The basic chemical reaction of CMP with ALP is depicted in Fig. S2(B). An increase in fluorescence response was observed with increasing ALP concentrations. This fits a normal enzyme reaction profile wherein the rate of product

formation is directly proportional to the concentration of the enzyme. In addition, copolymerization of the CMP monomer with acrylamide/bis-acrylamide monomers is expected to allow penetration of the protein into the hydrogel network without leaching CMP into the bulk solution. To verify this concept, the leaching behaviors of the CMH monomer and MUF, a similar compound without a copolymerization site, were compared in a washing experiment (Fig. S3). As expected, when the hydrogel formed was immersed in a buffer, the CMH dye molecule did not diffuse out of the hydrogel network, in contrast to MUF. This shows that successful copolymerization of CMP monomer with acrylamide monomer could be achieved.

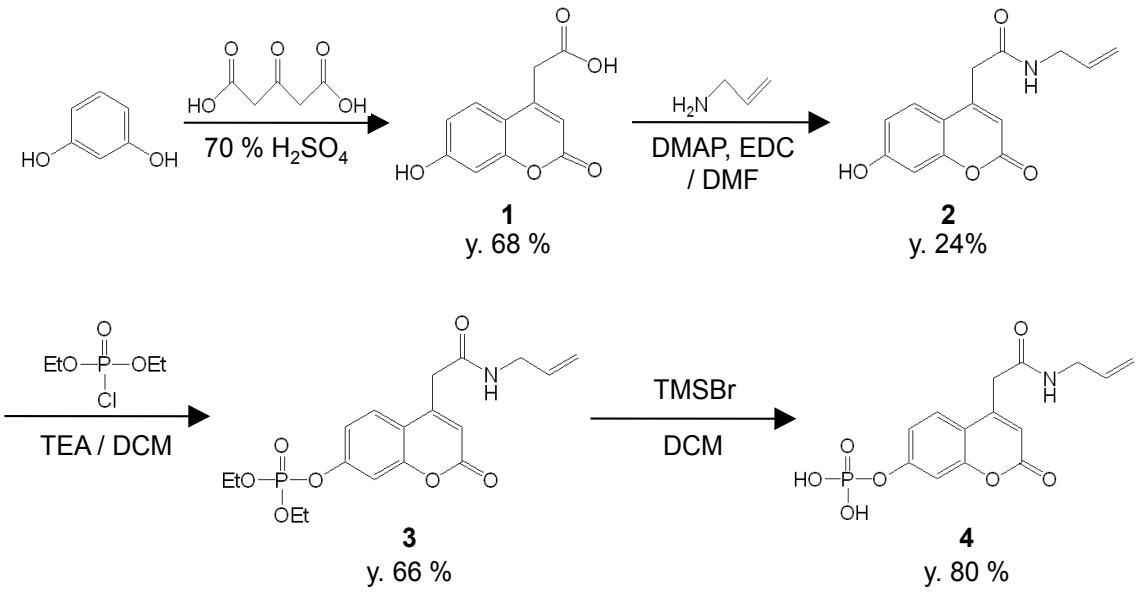

Figure S1. Synthesis of [2-(7-dihydroxyphosphoryloxy) coumarin-4-acetylamino]-propylene (CMP) monomer.

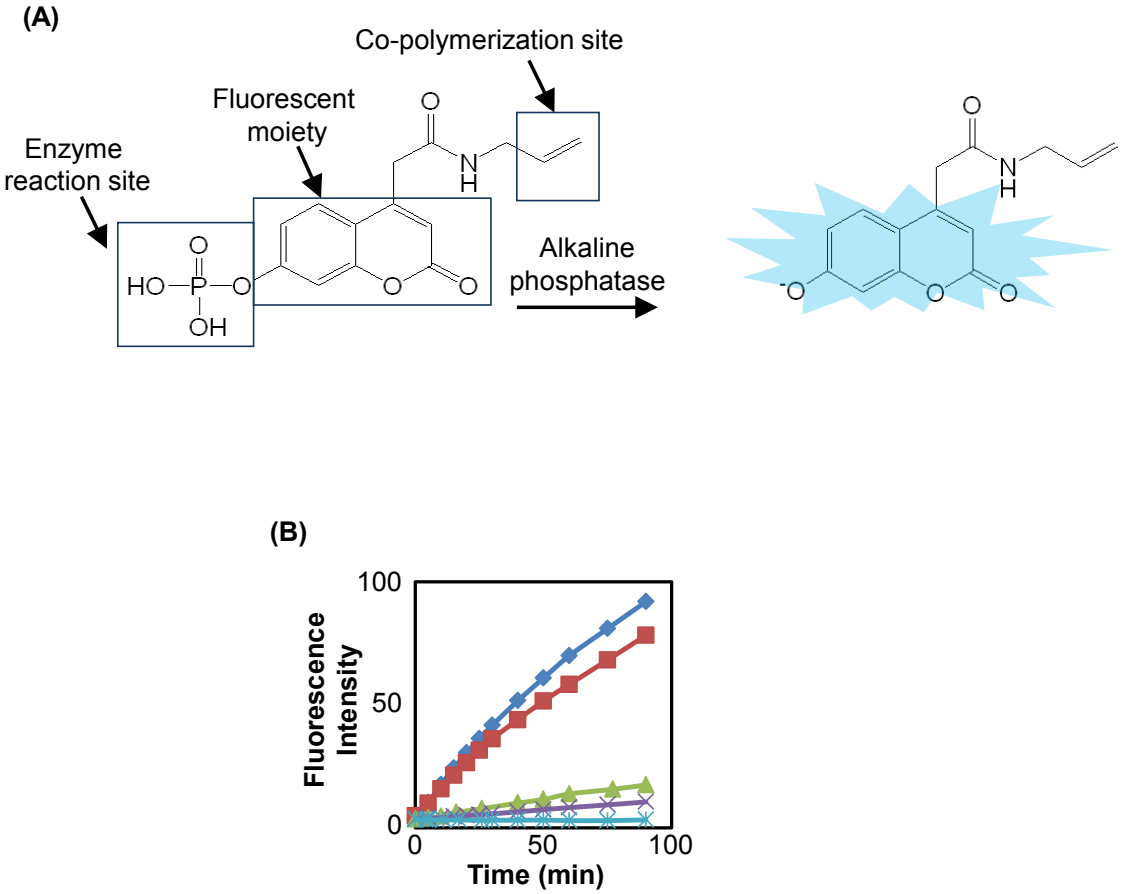

Figure S2. (A) Enzyme reaction of CMP monomer with alkaline phosphatase (ALP), **(B)** Monitoring of fluorescence response of CMP monomer (10^{-5} M) in the presence of various alkaline phosphatase concentrations (✳ 0, X 0.5, ▲ 1.0, ■ 5.0, and ◆ 7.5 mU/mL).

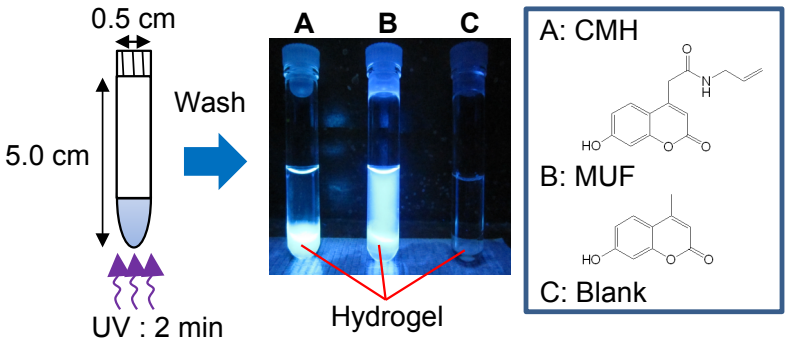

Figure S3. Comparison of three different hydrogels containing **(A)** CMH, **(B)** MUF and **(C)** without CMH or MUF (blank).

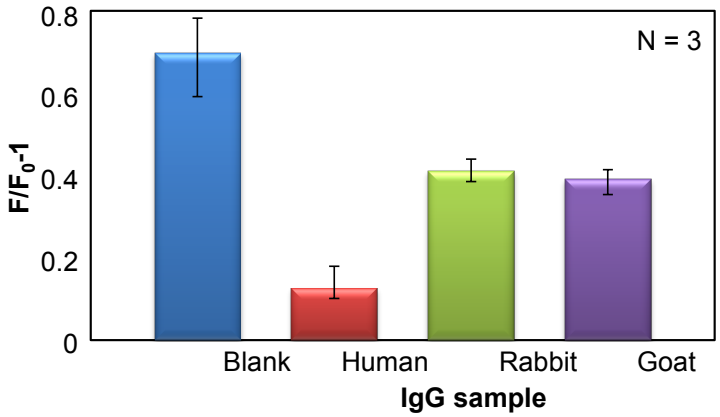

Figure S4. Hydrogel response to 50μg/mL of various antigens