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One-step biotinylation of cellulose paper by polymer-coating to prepare a paper-based analytical device

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Abstract Cellulose paper has strong potential as an analytical platform owing to its unique characteristics. In the present study, we investigated a procedure for functionalizing the surface of cellulose paper by dip-coating a mixture of a functional polymer and a perfluoroalkylated surfactant (surfactant 1). The functional polymer comprised a mixture of methyl methacrylate and poly(ethylene glycol) methacrylate monomers. The monomer ratio in the functional polymer affected the hydrophilicity and water absorbance of the cellulose paper after dip-coating. Furthermore, the presence of surfactant 1 during dip-coating promoted the surface segregation of poly(ethylene glycol) (PEG) moieties in the polymer, which enhanced the hydrophilicity, prevented nonspecific protein adsorption, and maintained the water absorbance of the dip-coated cellulose paper. Dip-coating with another functional polymer containing biotin groups produced a cellulose paper with a biotin-decorated surface in a one-step procedure. The displayed biotin groups immobilized avidin on the surface, and the PEG moieties in the polymer prevented nonspecific protein adsorption. We then immobilized a thrombin-binding DNA aptamer on the avidin-immobilized cellulose paper to prepare a paper-based analytical device. It is possible to visualize thrombin in model solutions and serum using the paper-based analytical device.

Keywords: DNA aptamer, protein in blood, immobilization, surface segregation, visual detection, water absorbance

Paper-based analytical devices have attracted a great deal of of attention as low-cost, portable, disposable, rapid and simple analytical platforms for point-of-care diagnostic assays, agriculture, food, and environmental sciences.[1-5] Currently, paper-based analytical devices are widely used in hospitals, physician's offices, and clinical laboratories for the qualitative and quantitative detection of specific antigens,[6] antibodies,[7] microorganisms,[8-11] and DNA/RNA.[12-14] A variety of biological samples are tested using paper-based analytical devices. These include urine,[15] saliva,[16] plasma,[17] serum,[18] whole blood[19-20] and other fluids.[21] The test results are indicated by changes in color, which depend on colorimetric reactions,[8-9, 11, 22] gold nanoparticles,[23-24] or fluorescent dyes.[7, 14, 25] It is often possible to detect these results directly with the naked eye, without the need for complex and expensive analytical equipment.

Despite these benefits, paper-based analytical devices still face major challenges, especially nonspecific protein/nonprotein adsorption (fouling) and limited strategies for selective immobilization of ligands/biomolecules.[26-28] Samples from a living bodies, agriculture, food and the environments contain a broad spectrum of compounds (e.g., proteins, polymers, sugars, lipids, and metal ions). In such complex mixtures, nonspecific protein adsorption significantly limits both the sensitivity and selectivity of analytical devices because of the drifting and denaturing of the detection ligands, the loss of target analytes during transport to the detection zone, and background noise from nonspecific protein adsorption.[29-31] To date, nonspecific protein adsorption has been suppressed by surface functionalization with hydrophilic polymers or proteins.[32-39] For example, poly(ethylene glycol) (PEG) provides protein-repellent properties on a wide variety of surfaces.[32, 40-41] Blocking

using bovine serum albumin (BSA) (or other kinds of proteins) also prevents nonspecific protein adsorption on a surface.[35-36, 42-43] However, it is still difficult to simultaneously achieve the prevention of nonspecific protein adsorption and the immobilization of ligands/biomolecules on a cellulose surface.

Coating with a functional polymer solution is a simple and versatile approach to functionalizing material surfaces, and does not require any specific apparatus.[43-45] In general, any hydrophilic moieties are likely to be buried in the bulk phase of the polymer when it is used as a coating solution. This occurs because of the surface segregation of the hydrophobic moieties in the polymer to minimize the surface energy.[46-50] We previously reported that the surface segregation of PEG moieties during dip-coating with a PEGylated copolymer can be controlled using a perfluoroalkylated surfactant (surfactant 1).[51]

In the present study, we aimed to functionalize cellulose paper surfaces by a simple dip-coating procedure using a PEGylated copolymer and surfactant 1. The PEGylated copolymer had PEG moieties and reactive sites (biotin). The coated paper surface simultaneously exhibited anti-fouling properties and reactivity for immobilizing a functional biomolecule, i.e., protein or DNA (Scheme 1). Finally, we demonstrated that the functionalized cellulose paper can be used as a paper-based analytical device for the detection of a disease-related enzyme in blood.

Experimental Section

Materials. The materials are described in the Supporting Information

Synthesis of PEGylated methacrylate-based copolymer (PMP, Scheme S1).

We synthesized a random copolymer composed of methyl methacrylate (MMA, 70 mol%) and poly(ethylene glycol) methyl ether methacrylate (PEGMA500, 30 mol%) by free-radical polymerization. MMA (70 mmol) and PEGMA500 (30 mmol) were dissolved in 50 mL of ethyl acetate in a glass vial. We bubbled N_2 through the solution, then initiated polymerization by adding azobisisobutyronitrile (AIBN, 0.20 mmol) and allowing the reaction to proceed at 70 °C for 20 h. After 20 h, we poured the solution into an excess of *n*-hexane at 25 °C, washed it twice with an excess of *n*-hexane, and dried it under vacuum for 24 h. The obtained random copolymer—poly(MMA-*r*-PEGMA) (monomer ratio of 7:3)—was named PMP(7:3). We synthesized two other random copolymers in a similar way: poly(MMA-*r*-PEGMA) (80 mol% : 20 mol%; named PMP(8:2)) and poly(MMA-*r*-PEGMA) (90 mol% : 10 mol%; named PMP(9:1)). PMP was identified by ^{1}H-NMR at 40 °C using an Avance-500 system (Bruker BioSpin GmbH, Rheinstetten, Germany) (Figure S1-S3) and an LC-2000 plus size-exclusion chromatography (SEC) system (JASCO, Tokyo, Japan) equipped with a 7.5 × 300 mm GF510 HQ SEC column (Showa Denko K.K., Tokyo, Japan) and an RI-2031 refractive index detector (JASCO, Tokyo, Japan), using tetrahydrofuran as an eluent (1.0 mL/min). ^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) = 4.12 (s, -CO-O-C*H*$_2$-CH$_2$-), 3.53–3.71 (m, -CH$_2$-O-CH$_2$-), 3.38 (s, -O-CH3), 1.73–2.08 (m, C-CH$_2$-), and 0.76–1.53 (m, C-CH$_3$). The ^{1}H-NMR measurements revealed that the monomer composition ratio (MMA : PEGMA500) of PMP(7:3) was 71:29. The SEC measurements suggested that PMP(7:3) had an M_n of 1.06 × 10^5 g/mol and an M_w/M_n of 1.41. The

data pertaining to the other poly(MMA-*r*-PEGMA) polymers are summarized in Table 1.

Table 1. Number-average molecular weights (M_n) and molecular weight distributions (M_w/M_n) of poly(MMA-*r*-PEGMA) (PMPs) synthesized in the present study.

Polymer	M_n	M_w/M_n	Monomer ratio m:n
PMP(9:1)	1.06×10^5	1.41	91 : 9
PMP(8:2)	6.27×10^4	2.00	84 : 16
PMP(7:3)	4.82×10^4	1.46	71 : 29

Synthesis of a perfluoroalkylated surfactant (surfactant 1, Scheme 1 and S2).

We dissolved octaethylene glycol monomethyl ether (1.5 mmol) and triethylamine (2.2 mmol) in 2.5 mL of dichloromethane. Tridecafluoroheptanoyl chloride (1.45 mmol) was added dropwise to the solution. The solution was stirred at room temperature overnight. We added water (3 mL) to the solution to extract octaethylene glycol monomethyl ether and triethylamine; this was repeated three times. We then collected and evaporated the dichloromethane phase, and dried the residue under vacuum overnight. The product (surfactant 1) was identified by ^{1}H-NMR (Figure S4) and matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI TOF-MS) using an ultrafleXtreme mass spectrometer (Bruker, Billerica, MA). ^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) = 4.52 (t, -CO-O-C*H*$_2$-CH$_2$-), 3.53–3.79 (m, -C*H*$_2$-O-C*H*$_2$-, -CH$_2$-C*H*$_2$-O-CH$_3$), 3.38 (s, -O-CH$_3$). MALDI TOF-MS (CHCA matrix), calcd for $C_{24}H_{35}O_{10}F_{13}Na$ ($M+Na^+$): 753.19. Found: 753.18.

Synthesis of biotin-containing PEGylated methacrylate monomer (Scheme S3).

We dissolved biotin-dPEG$_{11}$-NH$_2$ (500 mg) in a mixture of tetrahydrofuran (THF; 5.0 mL) and triethylamine (905 μL). Methacryloyl chloride (92.4 μL) was added dropwise to the solution over 10 min. The reaction mixture was stirred overnight at room temperature, and the resulting triethylamine hydrochloride was subsequently removed by filtration. We then washed the mixture three times with THF, evaporated the THF, and freeze-dried the product overnight. The product was identified by ^{1}H-NMR (Figure S5) and MALDI TOF-MS. ^{1}H -NMR (500 MHz, $CDCl_3$): δ (ppm) = 5.72, 5.32 (s, =CH_2), 4.50, 4.31 (m, −NH-C*H*(CH)-CH_2-), 3.53-3.70 (m, 48H, -CH_2-O-CH_2-), 3.14 (m, -CH_2-C*H*(CH)-S-), 2.73 (m, -CH-C*H*$_2$-S-), 2.22 (m, -CO-C*H*$_2$-CH_2-), 1.96 (s, CH_3-C-), 1.64-1.75 (m, -CH_2-C*H*$_2$-C*H*$_2$-CH-), and 1.44 (m, -CH_2-CH_2-C*H*$_2$-CH-). MALDI TOF-MS (DHB matrix), calcd for $C_{38}H_{71}N_4O_{14}S$ ($M+H^+$): 839.47. Found: 839.41.

Synthesis of biotin-termini PEGylated methacrylate-based copolymer (B-PMP, Scheme S4).

We synthesized a random copolymer comprising MMA (70 mol%) and a biotin-containing PEGylated methacrylate monomer (30 mol%) by free-radical polymerization. MMA (1.5 mmol) and the biotin-containing PEGylated methacrylate monomer (0.60 mmol) were dissolved in 5.0 mL of dimethyl sulfoxide (DMSO) in a glass vial. After bubbling N_2 through the solution, we initiated polymerization by adding AIBN (3.7 μmol) and allowing the reaction to proceed at 70 °C for 20 h. After 20 h, we poured the solution into excess water at room temperature, washed it twice with excess

water, and dried it under vacuum at room temperature for 24 h. We identified the synthesized copolymer—B-PMP(7:3)—by ^{1}H-NMR (Figure S6) and SEC. ^{1}H -NMR (500 MHz, $CDCl_3$): δ (ppm) = 4.51, 4.31 (m, −NH-C*H*(CH)-CH_2-), 3.39-3.74 (m, 48H, -CH_2-O-CH_2-), 3.14 (m, -CH_2-C*H*(CH)-S-), 2.75 (m, -CH-C*H*$_2$-S-), 2.22 (m, -CO-C*H*$_2$-CH_2-), 1.64–1.75 (m, -CH_2-C*H*$_2$-C*H*$_2$-CH-), 1.43 (m, -CH_2-CH_2-C*H*$_2$-CH-), and 0.76-1.07 (m, C-C*H*$_3$). The monomer composition ratio (MMA : biotin-containing methacrylate monomer) was 71:29, which was determined by the ^{1}H-NMR results. The SEC measurements suggested that the synthesized copolymer had an M_n of 2.0 × 10^4 g/mol and an M_w/M_n of 1.6.

Dip-coating cellulose papers in the synthesized polymers.

We cut 0.21 mm-thick cellulose papers into 1 cm × 1 cm pieces. First, PMP(7:3) was dissolved in 4.0 wt% ethyl acetate, and surfactant 1 (1.0 wt%) was added to the PMP(7:3) solution (PMP(7:3)/surfactant 1 solution). We also prepared a 4.0 wt% ethyl acetate solution containing PMP(7:3) without surfactant 1. B-PMP(7:3) was dissolved in 4.0 wt% DMSO. We then added surfactant 1 to a 1.0 wt% solution of B-PMP(7:3) (B-PMP(7:3)/surfactant 1 solution). The other polymer solutions were prepared in a similar manner. After stirring the polymer solutions overnight, we immersed the pieces of cellulose paper in the solutions for a few seconds (dip-coating), then withdrew them from the solutions over a few seconds, and dried them overnight under vacuum at 25 °C.

Examination of surface morphology.

We examined the surfaces of the paper substrates before and after coating using a JSM-7500F field-emission scanning electron microscope (FE-SEM; JEOL, Tokyo, Japan) operated at an accelerating voltage of 7 kV. Each paper substrate was sputter-coated with an ultra-thin osmium layer using a Neoc-STB osmium coater (Meiwafosis Co., Ltd., Tokyo, Japan) prior to the FE-SEM examination.

Water absorption and contact angle measurements.

The water absorption of the cellulose paper substrates (1 × 1 cm^2) was evaluated as follows. We weighed dry bare and coated cellulose paper substrates (1 × 1 cm^2), and immersed them in water for 30 s at 25 °C. The drips were then wiped the from paper substrates. After drying the substrates for 1 min at 25 °C in the open atmosphere, we weighed them to evaluate water absorption. The measurements were carried out in triplicate and error bars indicate standard deviations.

We measured the contact angles of water droplets on the paper substrates at 25 °C using a DMs-401 digital automated contact angle goniometer (Kyowa Interface Science Co., Ltd., Niiza, Japan). Images of the droplets were captured 1 s after placing a water droplet (5 μL) on the paper substrate. The images were used to determine the contact angles. We captured an image every 0.1 s for 1.5 s after a droplet was placed on the substrate, to measure the speed of penetration of water through the paper substrate.

Protein adsorption.

We immersed bare and coated paper substrates (1 × 1 cm^2) in 0.1 M phosphate buffer solution (pH 7.4, 4.0 mL) containing 1.0 mg/mL BSA for 0.5 h at 25 °C, then washed them three times with 5 mL of phosphate buffer. The amounts of BSA adsorbed on the paper substrates were measured using a Micro BCA™ Protein Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA). Briefly, we immersed the paper substrates in a mixture of a micro-BCA working reagent (1 mL) and a phosphate buffer (1 mL), then incubated them at 60 °C for 1 h. The absorbance of the solution was measured at 562 nm using a SH-9000 microplate reader (Corona Electric Co., Ltd., Hitachinaka, Japan). The measurements were carried out in triplicate and error bars indicate standard deviations.

Apart from the micro BCA assay, we immersed the bare and coated paper substrates (1 × 1 cm^2) in 0.1 M phosphate buffer solution (pH 7.4, 4.0 mL) containing 1.0 mg/mL of a BSA conjugate with fluorescein isothiocyanate (FITC-BSA) for 0.5 h at 25 °C, then washed them three times with 5 mL of phosphate buffer. We examined the surfaces of the dried paper substrates using an FL1000 confocal laser scanning microscope (CLSM; Olympus, Tokyo, Japan).

Immobilization of avidin on surfaces.

We immersed the bare and coated paper substrates (1 × 1 cm^2) in 0.1 M phosphate buffer solution (pH 7.4, 4.0 mL) containing 1.0 mg/mL FITC-BSA and 1.0 mg/mL avidin conjugated with tetramethylrhodamine isothiocyanate (TRITC-avidin) for 0.5 h at 25 °C, then washed them three times with 5 mL of phosphate buffer. The surfaces of the dried paper substrates were examined using

a CLSM.

Preparation of a paper-based analytical device.

We covered cellulose paper coated with PMP(7:3)/surfactant 1 (1 × 1 cm^2) with a polyester film, which had a hole (6 mm in diameter) in the center. The 6-mm diameter area served as a detection zone. First, we dissolved B-PMP(7:3) in 4.0 wt% DMSO. We then added 1.0 wt% surfactant 1 to the B-PMP(7:3) solution. The B-PMP(7:3)/surfactant 1 solution (3 μL) was dropped onto the detection zone and dried at 37 °C for 10 min (introduction of biotin parts onto the paper surfaces). A phosphate buffer solution (0.1 M, pH 7.4, 3 μL) containing 1.0 mg/mL NeutrAvidin was dropped onto the detection zone, then washed with excess water and dried at 37 °C for 10 min (NeutrAvidin was immobilized on the paper surface). We then dropped a phosphate buffer solution (3 μL) containing 50 μM biotinylated thrombin-binding DNA aptamer (15-mer + T_5, Table 2)[52] was dropped on the detection zone, washed it with excess water, and dried it at 37 °C for 10 min (the thrombin aptamer was immobilized on the paper surface via biotin–avidin interaction). The paper substrate with the biotinylated thrombin aptamer was used as a paper-based analytical device in the subsequent experiments.

Table 2. Oligo DNA sequences used in the present study.

Name	Sequence
Biotinylated thrombin aptamer	5' Biotin-T_5-GGTTGGTGTGGTTGG-3'
FITC-labeling thrombin	5' FITC-T_5-AGTCCGTGGTAGGGCAGGTTGGGGTGACT-

aptamer 3'

Detection of thrombin on a paper-based analytical device.

We dropped Dulbecco's phosphate-buffered saline (D-PBS(-), 3 μL) containing 50 mM K^+ and 0.2 mg/mL thrombin onto the detection zone of the paper-based analytical device, and washed it with an excess of D-PBS(-) containing 50 mM K^+. Then, we dropped a D-PBS(-) solution (3 μL) containing 50 mM K^+ and 50 μM FITC-conjugated thrombin-aptamer (29-mer + T_5, Table 2)[53] was dropped onto the detection zone, and washed it with an excess of D-PBS(-) containing 50 mM K^+. We recorded the fluorescence signal emitted by the paper-based analytical device in a UV CUBE (LC Science, Nara, Japan) equipped with a UV lamp (352 nm, 6 W, FL6BLB, Toshiba, Tokyo, Japan) and a digital camera (SX720 HS, Canon, Tokyo, Japan). The settings of the digital camera were as follows: exposure time 1/8 s; ISO 1600; and f-value 4.0. We measured the fluorescence intensity of the detection zone (on the 0–255 degree scale) using ImageJ image processing software (National Institutes of Health, Bethesda, MD).

Fetal bovine serum (FBS) containing 0.2 mg/mL thrombin was also tested as a model analyte. We added thrombin to FBS to produce a thrombin concentration of 0.2 mg/mL. The FBS solution (3 μL) containing 0.2 mg/mL thrombin was dropped onto the detection zone of the paper-based analytical device, and the procedure described above was followed.

The measurements were carried out in triplicate and error bars indicate standard deviations.

Results and discussion

Dip-coating and examination of the surface morphology.

We dip-coated a piece of cellulose paper (1 cm × 1 cm, 0.21 mm thick) in an ethyl acetate solution containing 4 wt% PMP(7:3) and 1 wt% surfactant 1, and dried it under vacuum. Weighing the substrates before and after dip-coating revealed that approx. 0.75 mg PMP(7:3) was put on a substrate and that a mixture (approx. 1.1 mg) of PMP(7:3)/surfactant 1 was put on a substrate. A PMP(7:3)/ethyl acetate solution was supposed to penetrate cellulose paper and PMP(7:3) was to cover fibers of cellulose paper. The anchoring and three-dimensional coverage would account for the stable adhesion of PMP to cellulose paper.

An FE-SEM was used to examine the bare and dip-coated paper substrates. Figure 1a–c shows that there were no significant differences in the surface morphologies of the paper substrates before and after dip-coating (at 250-fold magnification), and there were also no significant differences between the substrates coated with and without surfactant 1, although there seemed to be a thin layer on the dip-coated surface. The observation at 10,000-fold magnification showed that the surfaces of the fibers in the paper became smooth after dip-coating (Figure 1A–C). The presence of surfactant 1 did not make any visible difference to the fiber surfaces (Figure 1B and C). Figure 1α–γ shows the cross-sections of the paper substrates. No remarkable difference was found in the inner structure among bare and dip-coated paper substrates. These observations indicate the formation of a thin polymer layer on the paper fibers, which did not affect the network structure of the paper fibers.

Water absorption and water contact–angle measurements.

For paper-based analytical devices, the water absorption and water penetration of the cellulose paper are the important characteristics. A polymer coating often makes a cellulose paper hydrophobic, resulting in low or zero water absorption. We studied the water absorption of bare and dip-coated paper substrates. Figure 2 shows that bare cellulose paper absorbed water by as much as 14 mg/cm^2, whereas a paper substrate coated with poly(methyl methacrylate) (PMMA) absorbed a negligible amount of water. As the PMP content of the PEGMA500 composition increased, the water absorbance increased. The presence of surfactant 1 in the PMP(7:3) dip-coating increased its water absorbance by 25% compared with the water absorbance of the PMP(7:3) dip-coating without surfactant 1, and was comparable with that of a bare cellulose paper.

We then examined the water contact angles of dip-coated paper substrates (Figure 3A). A paper substrate with a dry bare surface had a water contact angle of approximately 25°, and the contact angle decreased rapidly to zero within 0.5 s, indicating rapid water absorption. Dip-coating with PMMA and PMPs (monomer ratio: 9:1 and 8:2) made the paper surface hydrophobic, and the contact angle remained approximately 100° over 1.5 s. When the cellulose paper was dip-coated with PMP(7:3), the contact angle decreased with time and reached 0° in 30 s, indicating that dip-coating with PMP(7:3) produces a relatively hydrophilic surface and that the paper substrate absorbs water gradually. Dip-coating with a PMP(7:3)/surfactant 1 mixture provided a hydrophilic surface, and the contact angle decreased rapidly. The contact angle decreased to almost zero within 1.5 s, indicating rapid water absorption. It should be noted that prior to the measurement, the cellulose paper was rinsed with a copious amount of deionized water to remove surfactant 1 from the surface, and

subsequently dried overnight at room temperature under vacuum. As we have reported previously, the presence of surfactant 1 induced the surface segregation of PEG moieties in PMP(7:3) when it was applied as a dip-coating and dried. This produced a dip-coated paper substrate with a hydrophilic surface.

Figure 3B shows the photo images of water droplets on the bare and dip-coated cellulose papers. As described above, the bare cellulose paper absorbed water rapidly. Water droplets on the PMMA and PMPs (monomer ratio: 9:1 and 8:2) had not changed 1.5 s after placement. Water droplets on surfaces dip-coated with PMP (7:3) collapsed with time, suggesting absorption by the dip-coated paper substrate. Paper substrates dip-coated with the PMP(7:3)/surfactant 1 mixture absorbed water droplets rapidly. These results suggest that the hydrophilic/hydrophobic balance of an amphiphilic polymer is important for the preparation of hydrophilic surfaces by dip-coating, and that the presence of surfactant 1 enhances the hydrophilicity and maintains the water absorbance of dip-coated paper substrates. The following experiments were carried out using PMP(7:3) to dip-coat cellulose paper.

Protein adsorption onto the dip-coated surfaces.

To evaluate the antifouling property of the surfaces, we conducted a protein adsorption assay using bovine serum albumin (BSA) as a model foulant. We immersed the bare paper and the dip-coated cellulose papers in a phosphate buffer solution containing 1.0 mg/mL BSA, then rinsed them with phosphate buffer. The amount of BSA adsorbed on the surfaces of the paper substrates was measured using a micro-BCA assay (Figure 4A). The bare paper surface adsorbed approximately 1.9 $\mu g/cm^2$ of

BSA, whereas the surface dip-coated with PMP(7:3) in the absence of surfactant 1 adsorbed 1.1 μg/cm^2 of BSA, indicating that PMP(7:3) alone reduces the nonspecific adsorption of BSA to some extent. Dip-coating with a mixture of PMP(7:3) and surfactant 1 reduced the BSA adsorption to approximately 0.2 μg/cm^2, which was only 1/10 that of bare cellulose paper and 1/5 that of the PMP-coated substrate. In the case of dip-coating with PMP(7:3) in the absence of surfactant 1, it is possible that some of the PEG moieties of the copolymer were buried in the bulk of the coated polymer layer owing to the surface segregation of hydrophobic moieties (methacrylate segments) in the PMP(7:3).[51] However, in the case of dip-coating with a mixture of PMP(7:3) and surfactant 1, the PEG moieties in the PMP(7:3) were segregated by surfactant 1, which induced the localization of the PEG moieties just beneath the outermost surface of the dip-coated layer. Because surfactant 1 is soluble in water, rinsing with water removed it to expose the PEG moieties of PMP(7:3) on the outermost surface. The observed antifouling property resulted from the presence of the PEG moieties on the surface.

Cellulose paper has a complicated fibrous structure that increases its actual surface area. The outermost surface of cellulose paper, which may be perceived by the naked eye, plays an important role in paper-based analytical devices. We then studied protein adsorption on the outermost surface using a fluorescently-labeled protein (FITC-BSA). Figure 4B shows the CLSM images of bare and dip-coated paper surfaces. The paper surface dip-coated with the PMP(7:3)/surfactant 1 mixture exhibited negligible fluorescence, suggesting a small amount of nonspecific protein adsorption, whereas the bare cellulose paper exhibited obvious fluorescence. The paper surface dip-coated with PMP(7:3) in the absence of surfactant 1 also exhibited relatively low fluorescence, which was higher

than that of the PMP(7:3)/surfactant 1 mixture, suggesting that dip-coating with a mixture of PMP(7:3)/surfactant 1 is effective for reducing nonspecific protein adsorption onto a paper surface. These results are consistent with the results of the micro BCA assay.

Immobilization of avidin onto the dip-coated surfaces.

To immobilize functional molecules on paper surfaces, we synthesized another copolymer, B-PMP(7:3). B-PMP(7:3) was composed of methyl methacrylate and methacrylate containing PEG with a biotin group at its terminus (Scheme S4). Biotin–avidin interaction is very strong ($K_a = 10^{-15}$ M) and stable under mild conditions.[54] Avidin has four binding sites for biotin, which is useful for the immobilization of proteins, vesicles, and nucleotides on surfaces. To evaluate the reactivity of the biotin displayed on the dip-coated surfaces, we immersed the bare and dip-coated cellulose papers in a phosphate buffer solution containing a 1.0 mg/mL mixture of FITC-BSA and TRITC-avidin, then rinsed them with phosphate buffer. Figure 5 shows the CLSM images of the bare and dip-coated paper surfaces. There was significant nonspecific adsorption of FITC-BSA (green fluorescence) and TRITC-avidin (red fluorescence) on the bare paper surface (Figure 5a and A). The paper surfaces dip-coated with copolymers (Figure 5b-d, B-D) exhibited less nonspecific adsorption of both FITC-BSA and TRITC-avidin compared with the bare paper surface. It should be noted that the paper surface coated with B-PMP(7:3) did not adsorb TRITC-avidin despite the biotin groups in the copolymer, indicating that the biotin groups were buried in the copolymer layer. However, Figure 5e and E reveal the presence of TRITC-avidin and the absence of FITC-BSA on the paper surface coated with the B-

PMP(7:3)/surfactant 1 mixture. These results indicate the successful and selective immobilization of avidin, and the presence of biotin groups on the paper surface coated with the B-PMP(7:3)/surfactant 1 mixture. Surfactant 1 induced the surface segregation of biotin-conjugated PEG moieties, and the display of biotin groups on the outermost surface of the dip-coated cellulose paper. It should be noted that some of biotin groups in B-PMP(7:3) were displayed on the outermost surface and that the others were buried in the inner of the B-PMP layer.

Application to diagnostic paper-based analytical devices.

Finally, we attempted to detect a protein of interest using the dip-coated paper substrate as the platform for an analytical device. We adopted thrombin from bovine serum as an analyte. Thrombin is a serine protease that plays a vital role in certain pathological and physiological processes, including inflammation, wound healing, and blood coagulation. Abnormal thrombin concentrations are related to many pathologic processes including leukemia, inflammation reactions, cardiovascular diseases, arterial thrombosis, and liver disease.[55-56] Thus, it is very important to monitor the thrombin level in blood for disease diagnosis.[57]

Figure 6 shows the design of our paper-based analytical device and the scheme for thrombin detection. We immobilized NeutrAvidin on the dip-coated paper substrates. A biotinylated thrombin-binding DNA aptamer (15-mer + T_5, Table 1) was then immobilized on the dip-coated paper surfaces via the biotin-avidin interaction (Figure 6B). An analyte solution (3 μL) was placed on the center of the paper, and excess PBS was used to remove unbound substances from the paper. A solution of

FITC-labeled thrombin-binding aptamer (29-mer + T_5, Table 1) was then placed on the paper to bind the 29-mer aptamer to the thrombin captured by the 15-mer aptamer on the paper substrate (Figure 6C). After rinsing, the fluorescence signal of the paper was recorded photographically.

Figure 7 shows images of the fluorescence signals from the bare and dip-coated paper substrates, and the fluorescence intensities quantified from the images. The bare cellulose paper itself and the papers coated with copolymers (substrates 1, 2, and 3) did not exhibit fluorescence. The control substrates (substrates 4, 5, and 6) were prepared in the absence of NeutrAvidin, the biotinylated thrombin-binding 15-mer aptamer, and thrombin, respectively. These surfaces exhibited weak or negligible fluorescence, which was not visible on the photo images. Other control substrates (substrates 7 and 8) were prepared with a PMP(7:3)/surfactant 1 mixture (no biotin) and with B-PMP(7:3) without surfactant 1 (no surface segregation of biotin groups). These surfaces exhibited some fluorescence, which was probably due to the nonspecific adsorption of thrombin. It should be noted that it was hard to detect the fluorescence in the photo images by the naked eye. The paper substrate coated with the B-PMP(7:3)/surfactant 1 mixture (substrate 9, the paper-based analytical device) exhibited strong fluorescence, which was also obviously visible on the photo image. The fluorescence intensity was more than twice that of the control surfaces (substrates 7 and 8). These results indicate that thrombin was successfully captured on the dip-coated paper substrate with immobilized NeutrAvidin, and its fluorescence was visible.

The investigations described above were carried out using a phosphate buffer solution containing thrombin, which did not contain other proteins or biomolecules. We then used fetal bovine serum

(FBS) containing thrombin as an analyte. FBS containing 0.2 mg/mL thrombin was applied to the paper-based analytical device and the fluorescence was recorded (substrate 10). The fluorescence signal was as high as that of substrate 9. The fluorescence was visible in the photo image, signifying the successful detection of thrombin in the serum using the paper-based analytical device.

We investigated the effect of the concentration of thrombin on the paper-based analytical device. Figure 8 shows images of the fluorescence signal and intensity curve at various concentrations. The photo images show that a thrombin concentration of more than 0.15 mg/mL can be detected by the naked eye. The fluorescence intensity increased with increasing thrombin concentration. The quantified intensity also reflects the increase in fluorescence intensity with increasing thrombin concentration. The quantified intensity allowed the detection of thrombin at low concentrations (0.05 and 0.1 mg/mL), which the naked eye was unable to detect. These results demonstrate that cellulose paper coated with the B-PMP(7:3)/surfactant 1 mixture can be used as an analytical device for the detection of thrombin.

CONCLUSIONS

Although there are a number of reports on the surface functionalization of cellulose paper, it has been a challenge to simultaneously achieve anti-fouling and water absorbance. In the present study, we succeeded in the surface functionalization of cellulose paper by dip-coating it in a PEGylated polymer to prepare a hydrophilic surface while preserving water absorbance. Dip-coating a cellulose paper with a PEGylated polymer containing biotin groups in the presence of surfactant 1 produced a

biotin-decorated surface in a one-step process, owing to the surface segregation of the biotin-conjugated PEG moieties. The displayed biotin groups immobilized avidin on the paper surface, while the PEG moieties in the polymer prevented nonspecific protein adsorption. A thrombin-binding DNA aptamer was then immobilized on the avidin-immobilized cellulose paper to prepare a paper-based analytical device. We finally succeeded in the visual detection of thrombin in model solutions and serum by the naked eye using the paper-based analytical device. The present approach for functionalizing a cellulose paper surface is simple and versatile. Moreover, it has the potential to extend the use of cellulose paper as an analytical platform, and can be applied to a variety of fabrics.

Supporting Information. Materials and Supplementary Results. 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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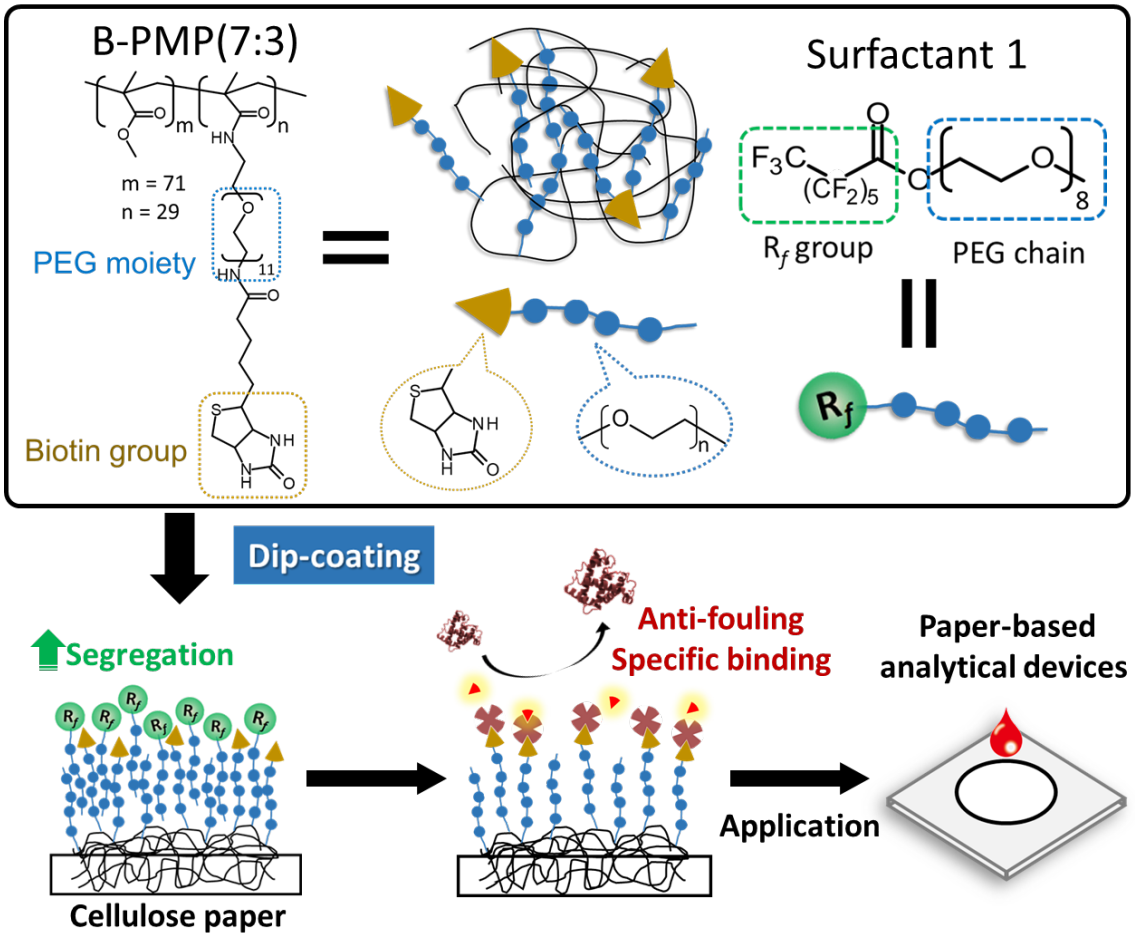
Based Thrombin Detection Using a DNA-Enzyme Conjugate Based on Histidine-Tag Chemistry. *Anal. Biochem.* **2012,** *421* (2), 541-546.

Scheme 1. Schematic illustration of the surface functionalization of cellulose paper by dip-coating with a PEGylated copolymer involving surface segregation of PEG moieties in the copolymer.



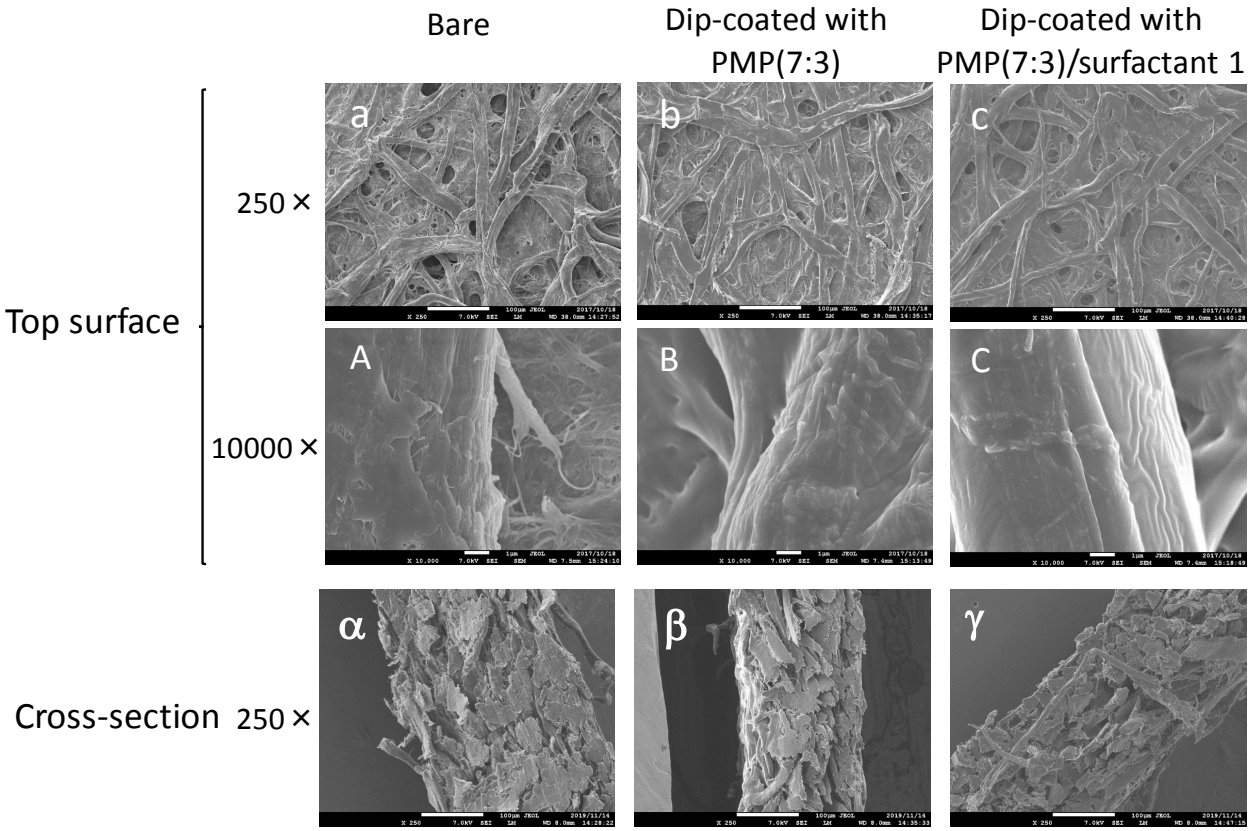


Figure 1. Field-emission scanning electron microscopy (FE-SEM) images of bare cellulose paper (a, A, α), paper substrates dip-coated with PMP(7:3) (b, B, β), and dip-coated with a PMP(7:3)/surfactant 1 mixture (c, C, γ). (a-c, α–γ) 250 × and (A-C) 10,000 × magnification. Scale bars represent 100 μm for (a-c, α–γ) and 1 μm for (A-C). (a-c, A-C) Top surfaces and (α–γ) cross-sections.

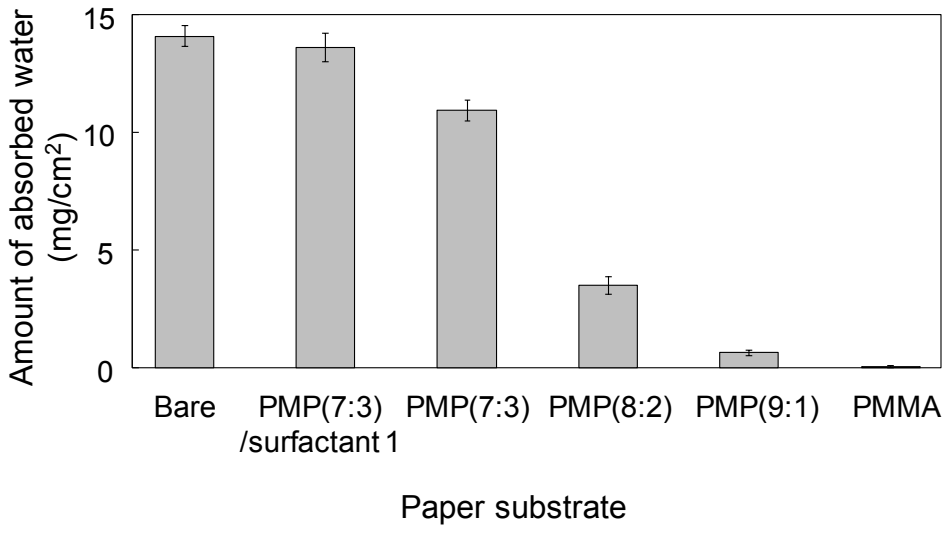


Figure 2. Water absorbance of cellulose paper substrates before and after dip-coating.

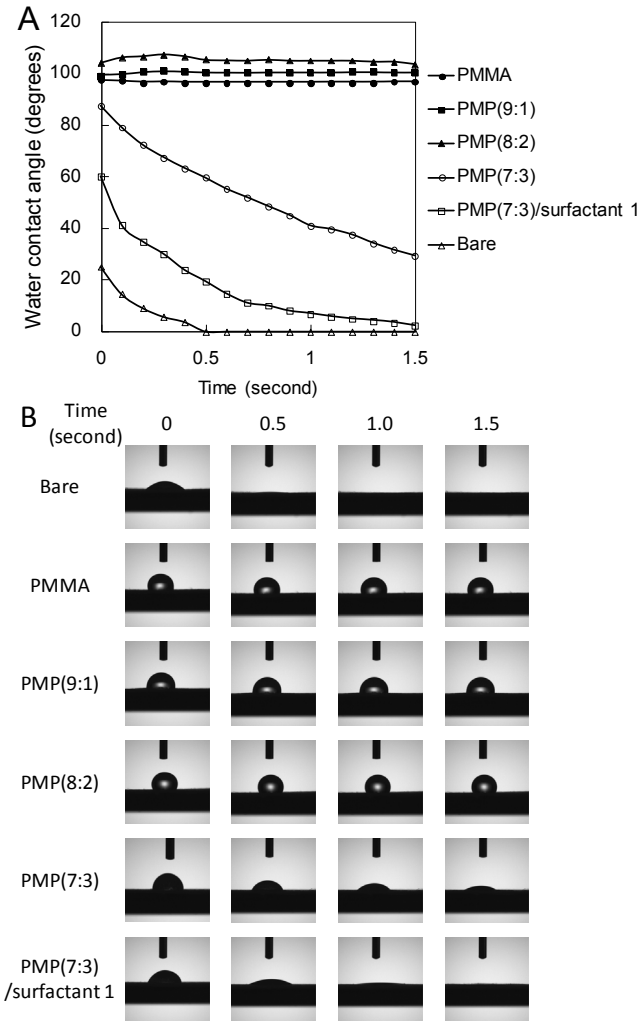


Figure 3. A) Contact angles of water droplets measured every 0.1 s. Contact angles on the bare and dip-coated cellulose papers. B) Images of water droplets on the surfaces taken every 0.5 s for 1.5 s.

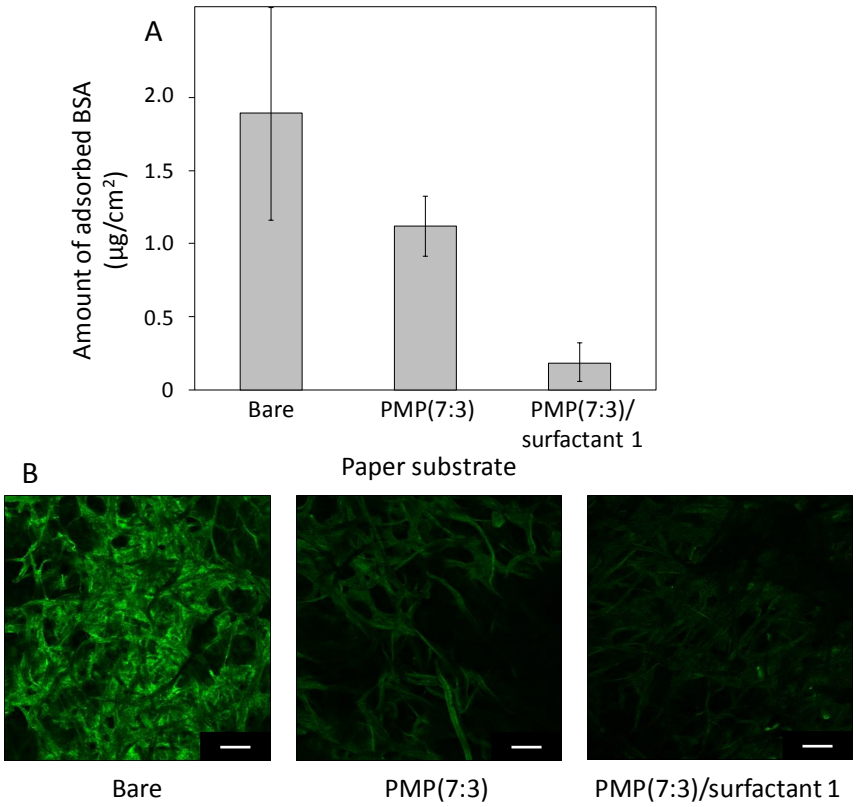


Figure 4. Adsorption of bovine serum albumin (BSA) on the bare and dip-coated cellulose papers. (A) The amount of BSA adsorbed on the bare paper, and on the paper substrates dip-coated with PMP(7:3) in the absence or presence of surfactant 1. (B) Confocal laser scanning microscope (CLSM) images of bare paper and paper substrates dip-coated with PMP(7:3) in the absence or presence of surfactant 1. The substrates were examined after FITC-BSA adsorption. Scale bars represent 100 μm.

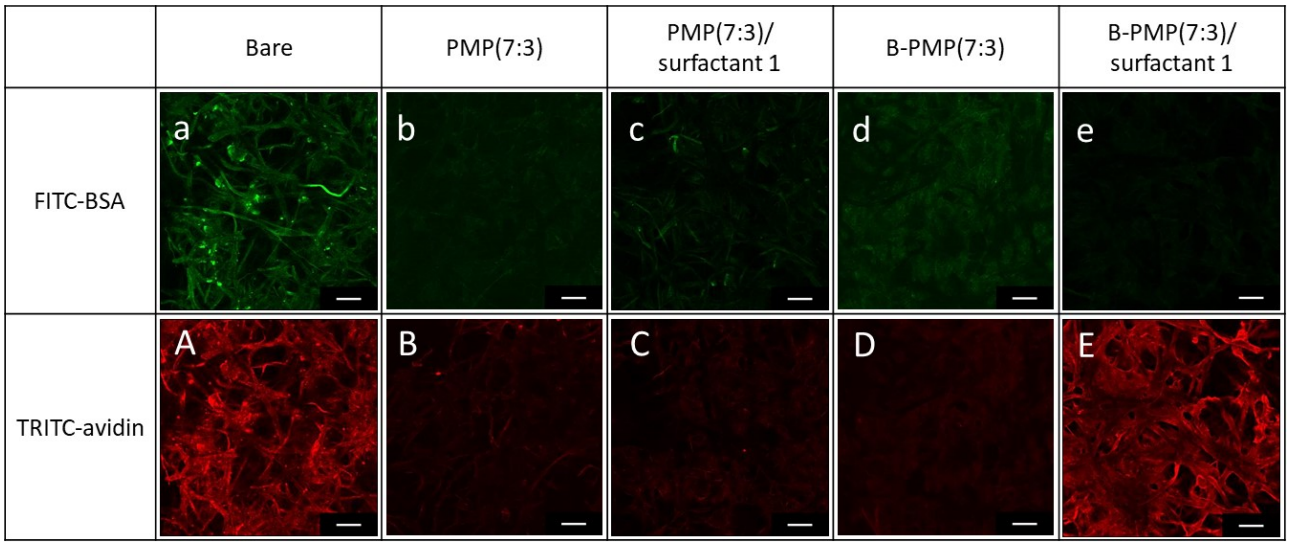


Figure 5. Confocal laser scanning microscope (CLSM) images of bare cellulose paper and paper substrates dip-coated with B-PMP(7:3) in the absence or presence of surfactant 1. The substrates were examined after adsorption of FITC-BSA and TRITC-avidin. Scale bars represent 100 μm. (a-e) Absorption of FITC-BSA. (A-E) Absorption of TRITC-avidin.

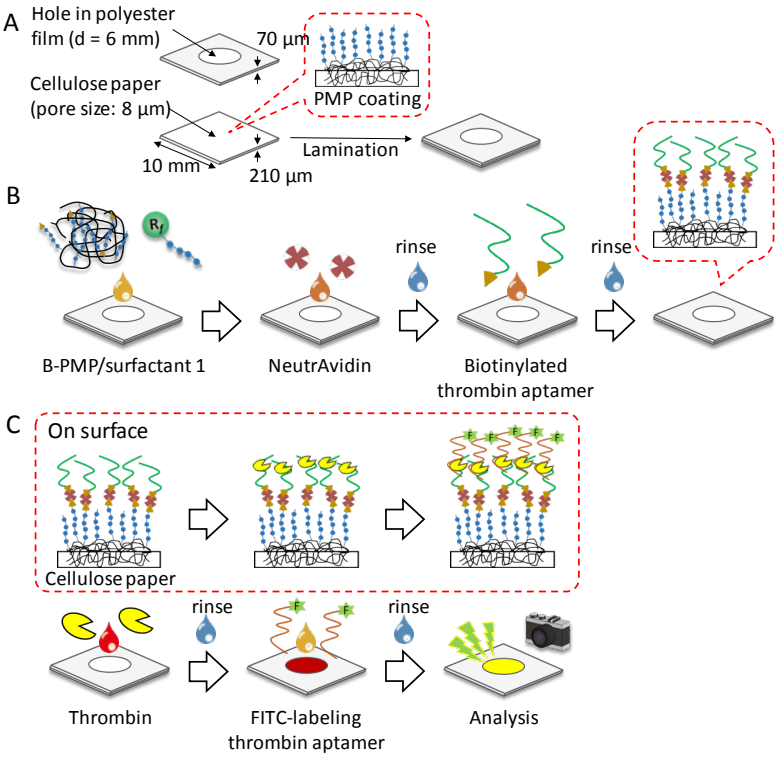


Figure 6. Schematic illustration of the preparation of the paper-based analytical device, and thrombin detection using it. A) Coating and lamination process of a cellulose paper using B-PMP(7:3)/surfactant 1 and a polyester film. B) Immobilization of a biotinylated thrombin-binding DNA aptamer on the dip-coated cellulose paper. C) Thrombin detection.

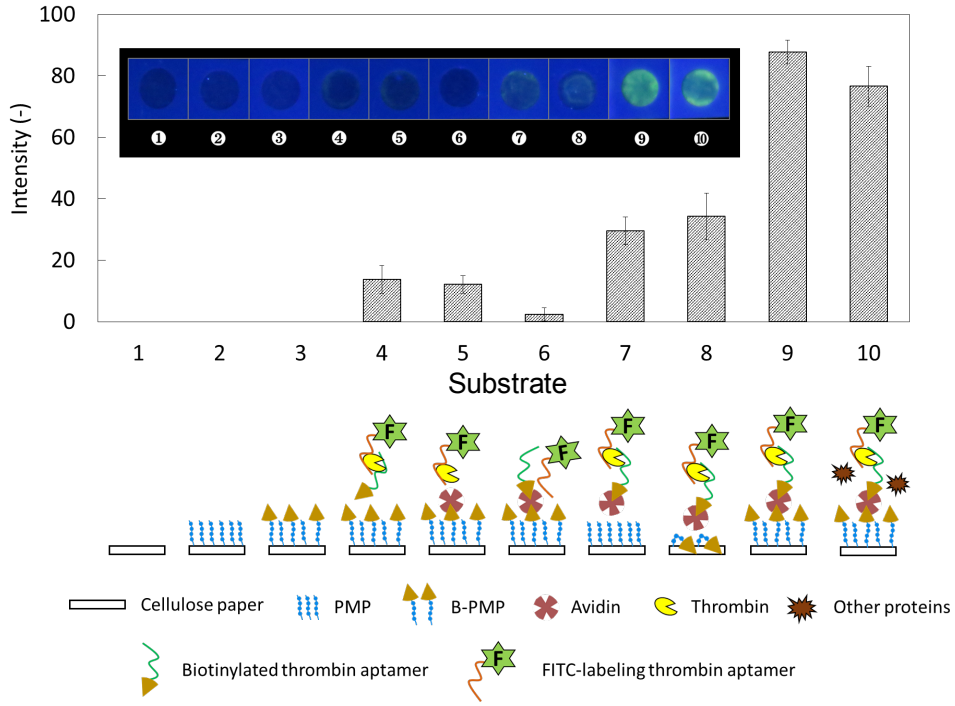


Figure 7. Fluorescence measurements of bare and dip-coated paper surfaces during thrombin detection. The insets are photographic images of the bare and dip-coated cellulose paper substrates. A phosphate buffer solution (3 μL) containing 0.2 mg/mL thrombin was placed on the center of a paper substrate. The wavelength of the UV lamp was 352 nm. The background fluorescence (bare substrate) was subtracted from the fluorescence of each sample.

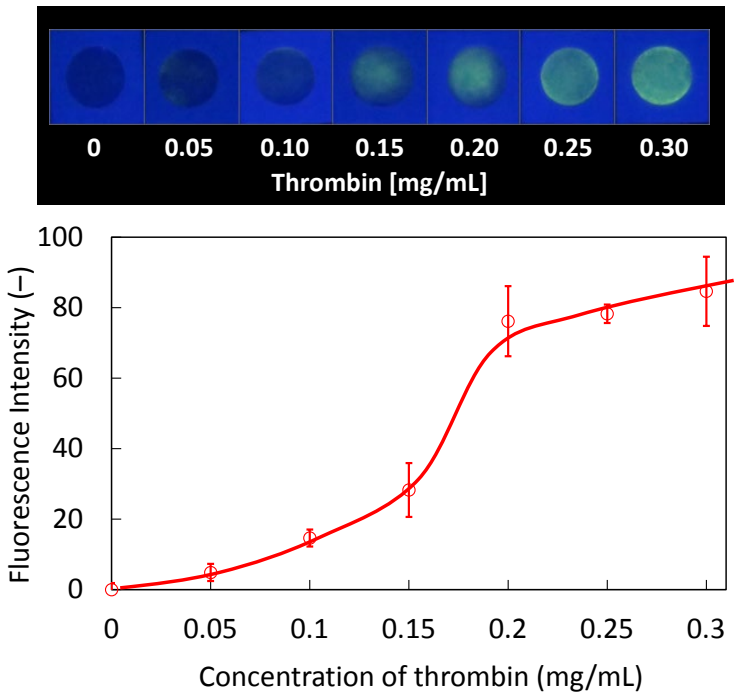
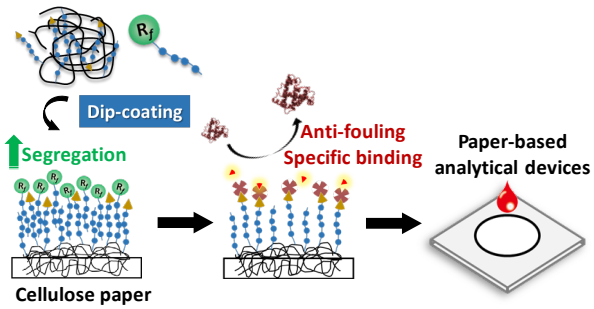


Figure 8. Photo images of paper-based analytical devices (upper panel) and fluorescence signals (lower panel) at various thrombin concentration. The wavelength of the UV lamp was 352 nm.

For TOC only


Rf
Dip-coating
Segregation
Anti-fouling
Specific binding
Paper-based
analytical devices
Cellulose paper

Supporting information

One-step biotinylation of cellulose paper by polymer-coating to prepare a paper-based analytical device

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Materials.

Poly(ethylene glycol) methyl ether methacrylate (PEGMA 500), poly(methyl methacrylate) (PMMA), and fetal bovine serum (FBS) were purchased from Sigma (St. Louis, MO). Biotin-dPEG$_{11}$-NH$_2$ was purchased from Quanta BioDesign, Ltd. (Plain City, OH). Octaethylene glycol monomethyl ether was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Bovine serum albumin (BSA) conjugate with fluorescein isothiocyanate (FITC-BSA) and tridecafluoroheptanoyl chloride were purchased from Aldrich (Milwaukee, WI). Avidin conjugated with tetramethylrhodamine isothiocyanate (TRITC-avidin) was purchased from Vector Laboratories (Burlingame, USA). NeutrAvidin was purchased from Thermo Fisher Scientific Inc. (Waltham, MA). Synthesized DNA oligonucleotides were purchased from Tsukuba Oligo Service Co., Ltd. (Tsukuba, Japan). Cellulose paper with a diameter of 150 mm (Whatman quantitative filter paper, ashless, grade 40, pore size 8 μm) was purchased from GE Healthcare Life Sciences (Pittsburgh, PA). Other chemicals were purchased from Wako Pure Chemical Industries. Methyl methacrylate (MMA) and PEGMA500 were used after they had passed through a column packed with activated alumina to remove the stabilizer.

Characterization of synthesized polymers and monomers

PEGylated methacrylate-based copolymer (PMP, Scheme S1): ^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) = 4.12 (s, -CO-O-C*H*$_2$-CH$_2$-), 3.53–3.71 (m, -CH$_2$-O-CH$_2$-), 3.38 (s, -O-CH3), 1.73–2.08 (m, C-CH$_2$-), and 0.76–1.53 (m, C-CH$_3$). The ^{1}H-NMR measurements revealed that the monomer

composition ratio (MMA : PEGMA500) of PMP(7:3) was 71:29. The SEC measurements suggested that PMP(7:3) had an M_n of 1.06×10^5 g/mol and an M_w/M_n of 1.41. The data pertaining to the polymers synthesized in the present study are summarized in Table S1.

A perfluoroalkylated surfactant (surfactant 1, Scheme 1 and S2): ^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) = 4.52 (t, -CO-O-C*H*$_2$-CH$_2$-), 3.53–3.79 (m, -C*H*$_2$-O-C*H*$_2$-, -CH$_2$-C*H*$_2$-O-CH$_3$), 3.38 (s, -O-CH$_3$). MALDI TOF-MS (CHCA matrix), calcd for $C_{24}H_{35}O_{10}F_{13}Na$ ($M+Na^+$): 753.19. Found: 753.18.

Biotin-containing PEGylated methacrylate monomer: ^{1}H -NMR (500 MHz, $CDCl_3$): δ (ppm) = 5.72, 5.32 (s, =CH$_2$), 4.50, 4.31 (m, −NH-C*H*(CH)-CH$_2$-), 3.53-3.70 (m, 48H, -CH$_2$-O-CH$_2$-), 3.14 (m, -CH$_2$-C*H*(CH)-S-), 2.73 (m, -CH-C*H*$_2$-S-), 2.22 (m, -CO-C*H*$_2$-CH$_2$-), 1.96 (s, CH$_3$-C-), 1.64-1.75 (m, -CH$_2$-C*H*$_2$-C*H*$_2$-CH-), and 1.44 (m, -CH$_2$-CH$_2$-C*H*$_2$-CH-). MALDI TOF-MS (DHB matrix), calcd for $C_{38}H_{71}N_4O_{14}S$ ($M+H^+$): 839.47. Found: 839.41.

Biotin-termini PEGylated methacrylate-based copolymer (B-PMP(7:3), Scheme S4): ^{1}H -NMR (500 MHz, $CDCl_3$): δ (ppm) = 4.51, 4.31 (m, −NH-C*H*(CH)-CH$_2$-), 3.39-3.74 (m, 48H, -CH$_2$-O-CH$_2$-), 3.14 (m, -CH$_2$-C*H*(CH)-S-), 2.75 (m, -CH-C*H*$_2$-S-), 2.22 (m, -CO-C*H*$_2$-CH$_2$-), 1.64–1.75 (m, -CH$_2$-C*H*$_2$-C*H*$_2$-CH-), 1.43 (m, -CH$_2$-CH$_2$-C*H*$_2$-CH-), and 0.76-1.07 (m, C-C*H*$_3$).

Table S1. Number-average molecular weights (M_n) and molecular weight distributions (M_w/M_n) of poly(MMA-*r*-PEGMA) (PMPs) and biotin-termini PEGylated methacrylate-based copolymer (B-PMP(7:3)) synthesized in the present study.

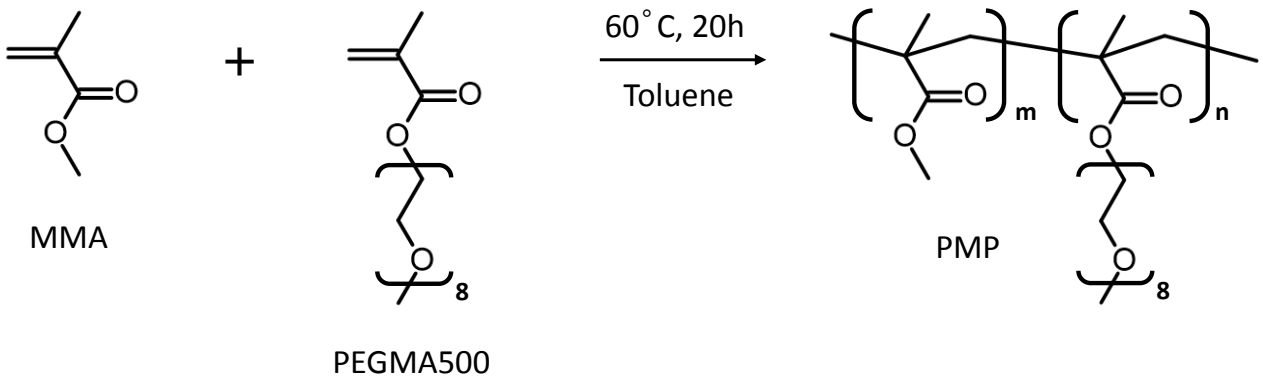
Polymer	M_n	M_w/M_n	Monomer ratio* m:n
PMP(9:1)	1.06×10^5	1.41	91 : 9
PMP(8:2)	6.27×10^4	2.00	84 : 16
PMP(7:3)	4.82×10^4	1.46	71 : 29
B-PMP(7:3)	2.0×10^4	1.64	71 : 29

*The monomer composition ratio (MMA : biotin-containing methacrylate monomer) was 71:29, which was determined by the 1H-NMR results.

Table S2. Oligo DNA sequences used in the present study.

Name	Sequence
Biotinylated thrombin aptamer	5' Biotin-T_5-GGTTGGTGTGGTTGG-3'
FITC-labeling thrombin aptamer	5' FITC-T_5-AGTCCGTGGTAGGGCAGGTTGGGGTGACT-3'

Scheme S1 Synthesis of poly(MMA-*r*-PEGMA) (PMP).



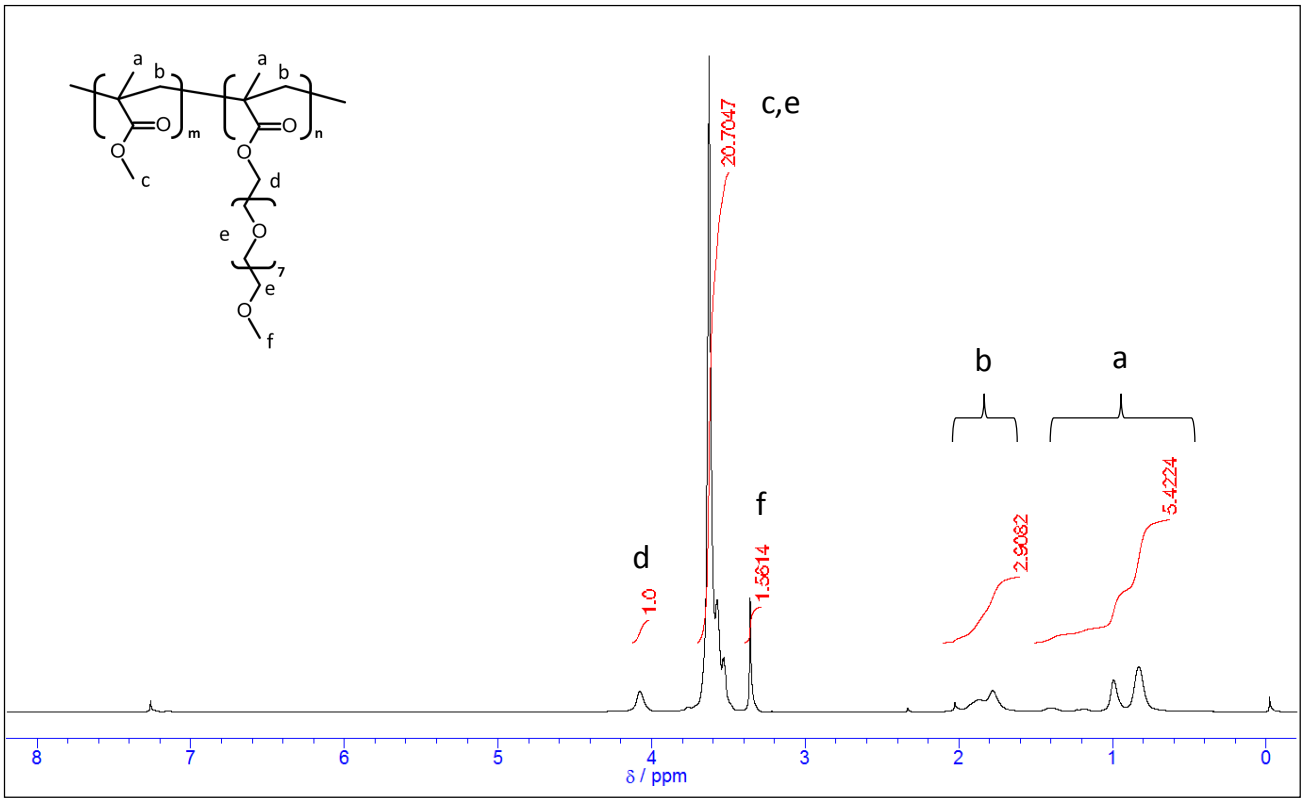


Figure S1 ^{1}H-NMR chart of PMP(7:3). The chemical shifts of a-f were assigned to ^{1}H labeled in the molecular structure (inset).

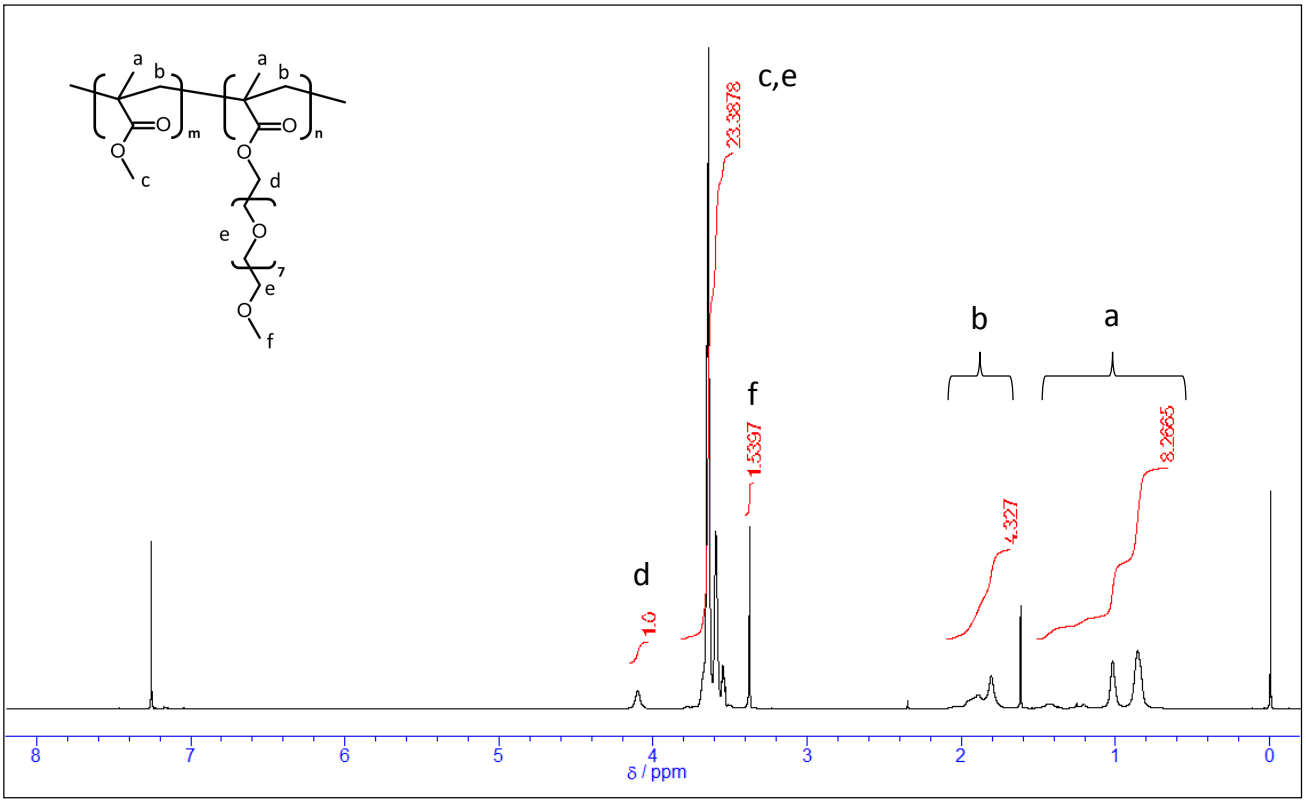


Figure S2 ^{1}H-NMR chart of PMP(8:2). The chemical shifts of a-f were assigned to ^{1}H labeled in the molecular structure (inset).

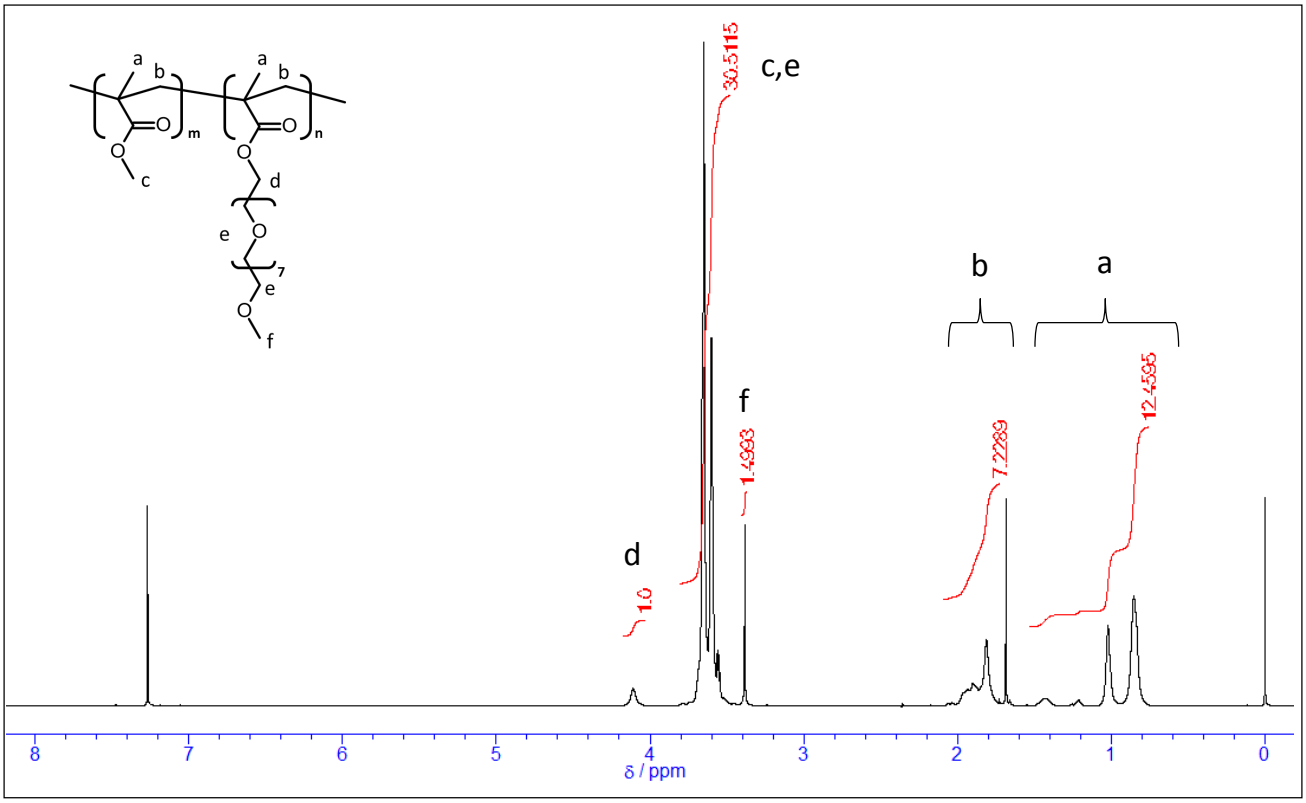
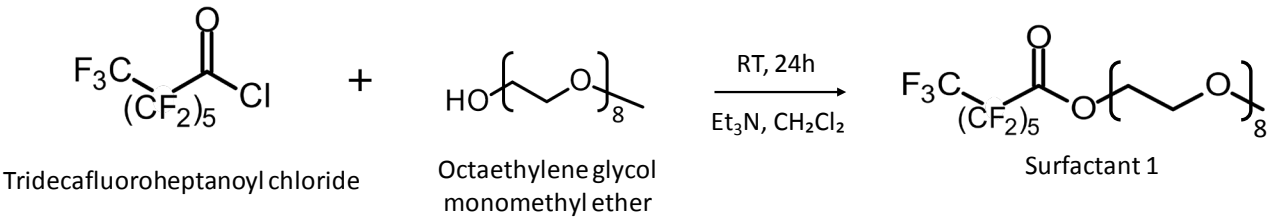


Figure S3 ^{1}H-NMR chart of PMP(9:1). The chemical shifts of a-f were assigned to ^{1}H labeled in the molecular structure (inset).

Scheme S2 Synthesis of a perfluoroalkylated surfactant (surfactant 1).



Tridecafluoroheptanoyl chloride

Octaethylene glycol monomethyl ether

Surfactant 1

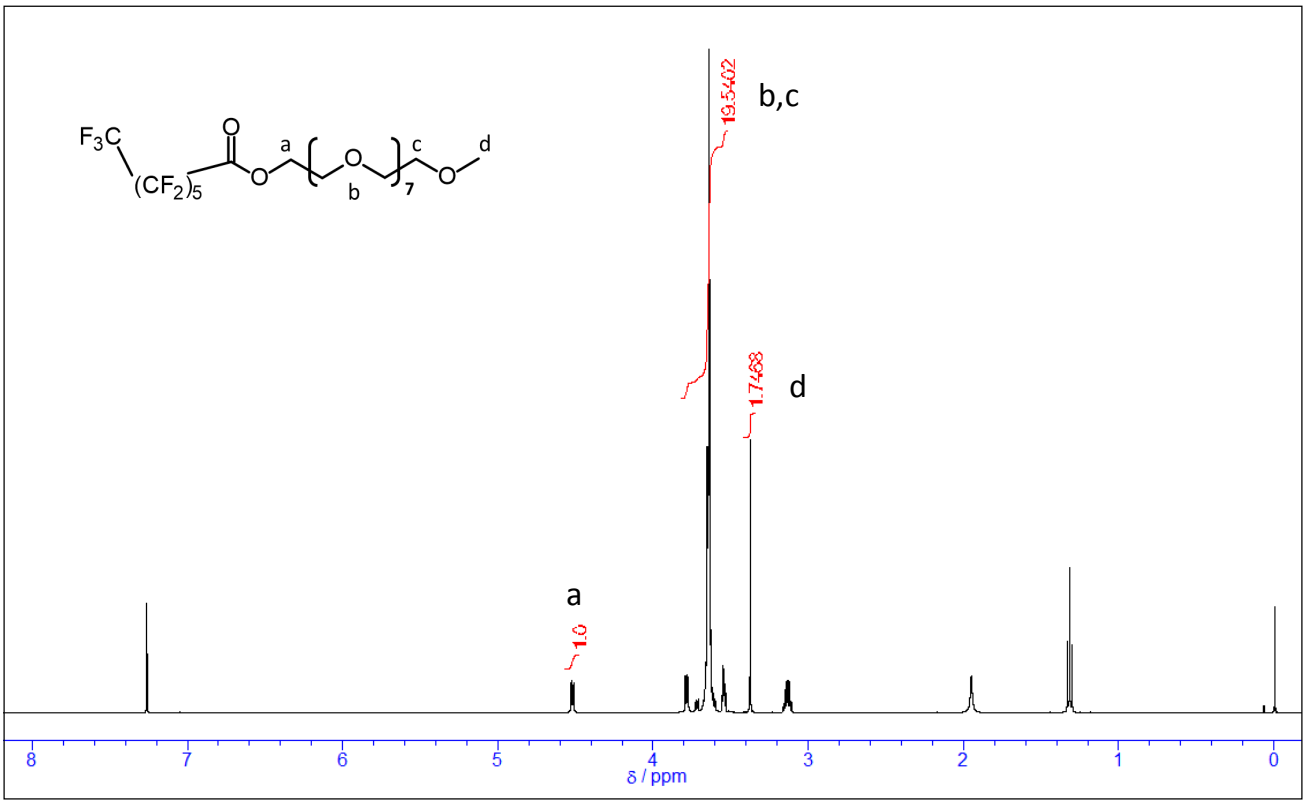
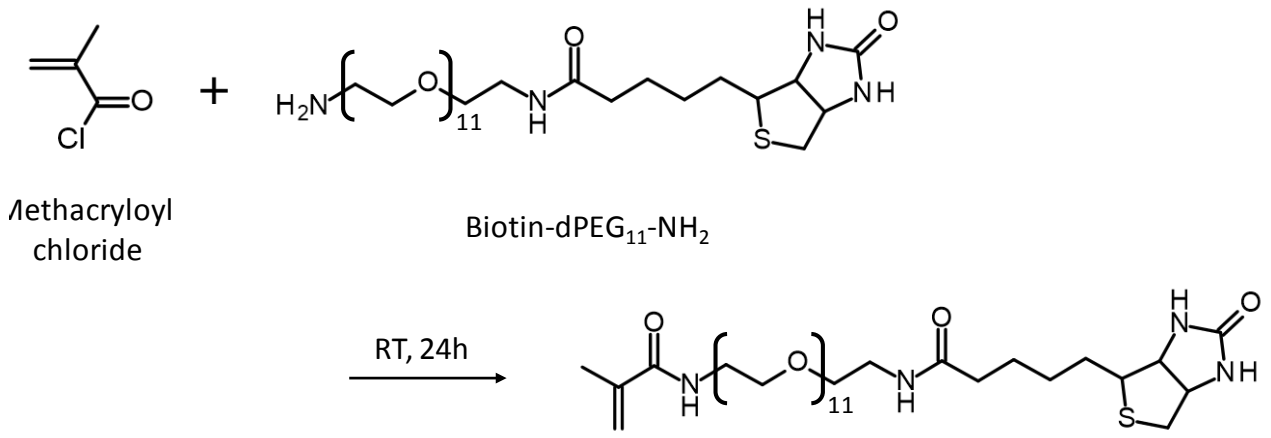


Figure S4 ^{1}H-NMR chart of a perfluoroalkylated surfactant (surfactant 1). The chemical shifts of a-d were assigned to ^{1}H labeled in the molecular structure (inset).

Scheme S3 Synthesis of biotin-containing PEGylated methacrylate monomer.



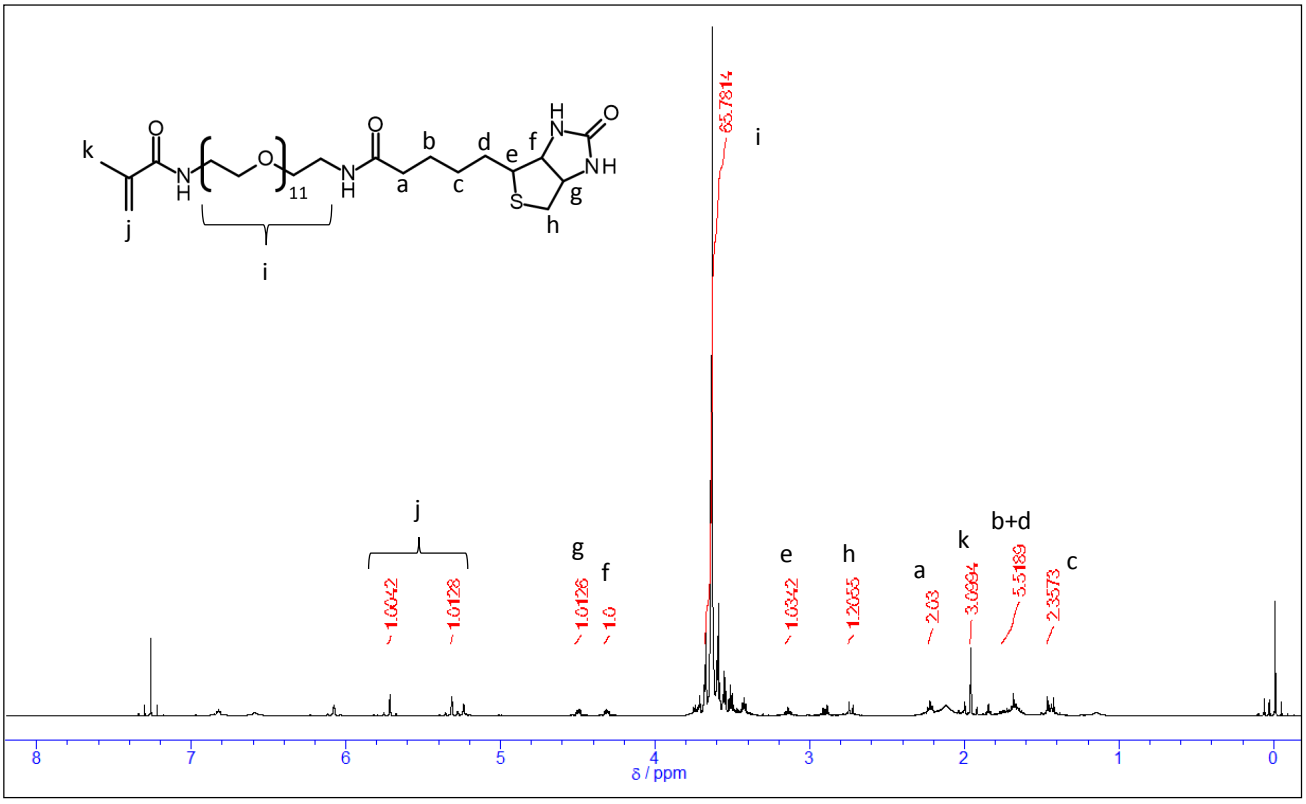
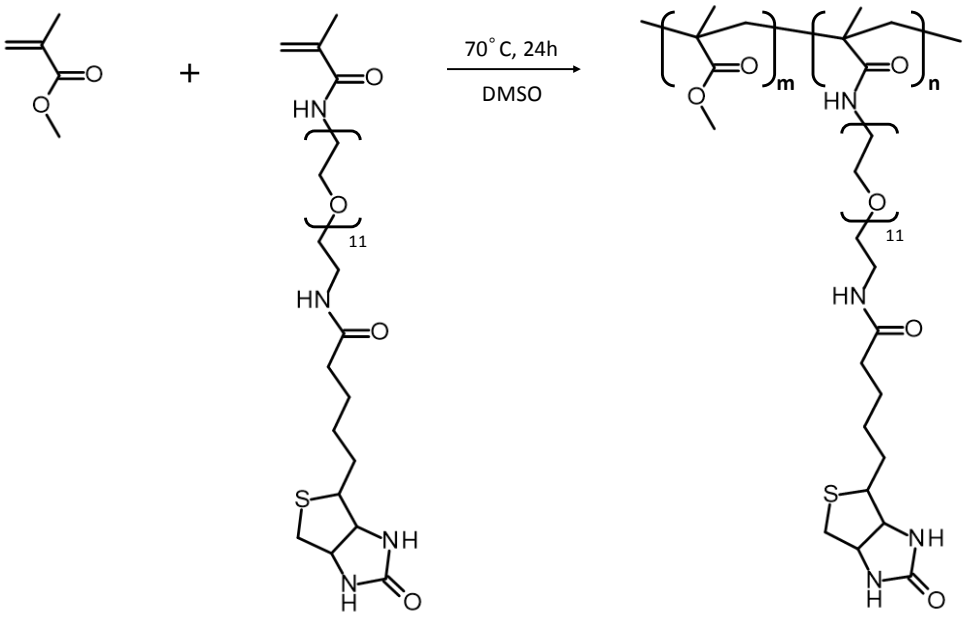


Figure S5 [1]H-NMR chart of the biotin-containing PEGylated methacrylate monomer. The chemical shifts of a-k were assigned to [1]H labeled in the molecular structure (inset).

Scheme S4 Synthesis of biotin termini-PEGylated, methacrylate-based copolymer (B-PMP(7:3)).



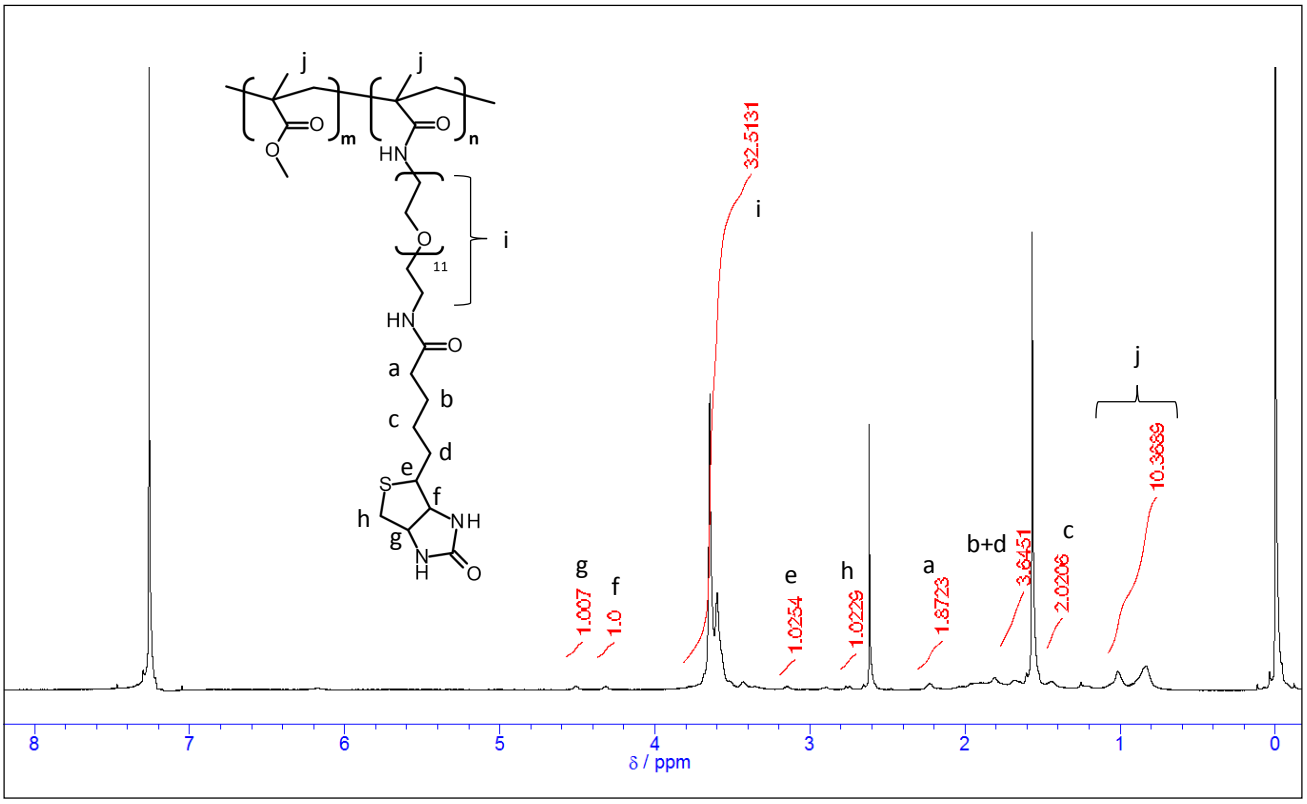


Figure S6 ^{1}H-NMR chart of biotin termini-PEGylated, methacrylate-based copolymer (B-PMP). The chemical shifts of a-j were assigned to ^{1}H labeled in the molecular structure (inset).