Electronic Supplementary Material (ESI) for Analytical Methods.

Supporting Information

Design of a highly sensitive and versatile membrane-based immunosensor using the Cu-free click reaction

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Materials

The track-etched polyethylene terephthalate (PET) substrates (for IL-6 detection: porosity = 19.6%, pore diameter = 1 μm, and thickness = 22 μm, for measurement of water contact angle: porosity = 18.8%, pore diameter = 0.4 μm, and thickness = 23 μm) were purchased from it4ip (Louvain-la-Neuve, Belgium). Porous high-density polyethylene (PE) membrane (porosity = 50%, average pore diameter = 200 nm, and thickness = 25 μm) was supplied by Asahi-Kasei (Tokyo, Japan). Glycidyl methacrylate (GMA), sodium azide, ammonium chloride, and sulfuric acid were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). The inhibitor remover of GMA was purchased from Sigma-Aldrich. Sodium dodecyl sulfate (SDS) and (1*R*,8*S*,9s)-bicyclo[6.1.0]non-4-yn-9-ylmethyl *N*-succinimidyl carbonate (BCN-NHS) were purchased from TCI (Tokyo, Japan). Human IL-6 antibody (capture antibody), recombinant human IL-6 protein (antigen), streptavidin-HRP, and human/primate IL-6 biotinylated antibody (secondary antibody) were purchased from R&D systems (Minneapolis, Minnesota, USA). TMB solution and FITC-labeled anti-BSA antibody were purchased from Bethyl Laboratories. Well plates (24/96) were purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA).

Plasma-induced graft polymerization of GMA

The pristine porous membranes were modified using GMA through plasma-induced graft polymerization (PIGP; the equipment structure is shown in Figure S1)[1, 2]. The porous PE membrane or track-etched PET membrane was cut into 3 × 3 cm^2 squares and rinsed with hexane. The membranes were then placed in a glass ampoule and treated with argon plasma for 1 min at a pressure of 10 Pa and a power of 30 W. The plasma-treated membranes were exposed to air for 1 min to generate peroxide groups on its pore surface. The membranes were then immersed in an aqueous solution of 0.5-wt% GMA monomer with 2.0-wt% SDS as the surfactant. The membrane-immersed solution was degassed via N_2 bubbling for 10 min and was placed in a water bath shaker (TAITEC MM-10) at 80 °C for 30 min. Active radical sites on the pore surface were formed via thermal cleavage of the peroxide groups, and then, the initiator radicals initiated a graft polymerization reaction. After polymerization, the membranes were washed overnight using a 50% aqueous ethanol solution. The grafting percentage of the membrane φ is calculated as follows:

$$\varphi[\%] = \frac{W_{graft} - W_{sub}}{\rho_{polym} \cdot V_{pore}} \times 100 \qquad \text{(eq. S1)}$$

Here, W_{sub} (g) and W_{graft} (g) represent the dry weight of the membrane before and after graft polymerization, respectively. ρ_{polym} (g/cm^3) denotes the density of the grafted polymer and was assumed to be 1.0 g/cm^3. V_{pore} (cm^3) represents the pore volume of the membrane. The grafting ratio, which is estimated from the membrane weight, correlates with the polymer-derived peak in

FT-IR (Figure S2); therefore, both FT-IR analysis and weight changes were used in determining the grafting ratio to increase reliability for low grafting ratios.

Introduction of azide group and diol into the grafted GMA

Six millimoles of sodium azide and six millimoles of ammonium chloride were dissolved in 20 ml of *N*,*N*-dimethylformamide (DMF). The membranes obtained after PIGP were immersed in this solution, and the azidation of the epoxy group in PGMA was performed for 2 h at 60 °C. The membranes were then rinsed using a 50% aqueous ethanol solution. After azidation, to remove the unreacted epoxy groups and introduce diol groups into the pore, the membranes were additionally immersed in 1-M sulfuric acid overnight at 30 °C. At each step, the membranes were analyzed using Fourier transform infrared (FTIR) spectroscopy.

Measurement of water contact angle

The increase in hydrophilicity of the diol-formed membrane interface was confirmed by measuring the water contact angle. This measurement was performed using a pristine PE substrate and the membrane after azidation and ring-opening reaction. PET substrate with a pore size of 0.4 µm were used because wicking prevents proper measurement when membrane with 1 µm pore size was used. Water contact angle measurements were performed with a Drop Dhape Analyzer (DSA100, KRÜSS) and the contact angle was read when a 2.67 µl of water was dropped.

Coupling of capture antibody and cyclooctyne

To 1 ml of the capture-antibody stock solution (1 mg/ml), 0.2 mg of BCN-NHS was added, and the reaction was performed for 1 h at room temperature (~25°C). In this condition, the ratio of BCN-NHS and antibody was ~100:1. After the reaction, the solution was purified using a desalting column, the obtained antibody solution was then concentrated through ultrafiltration. The antibody concentration was determined based on the peak observed at 280 nm via a UV–vis spectrophotometer (Figure S4).

Immobilization of capture antibody via Cu-free click chemistry

Azide/diol-modified membranes were cut into circles of 1-cm diameter, and each membrane was placed in the wells of a 24-well microplate. Subsequently, the capture antibody solution after modification with cyclooctyne (200 µg/ml, 80 µl) was dropped onto the membrane. The membranes were incubated at room temperature to immobilize the antibody through chemical binding using Cu-free click chemistry. After incubation, the membranes were briefly rinsed using pure water and used in IL-6 detection tests.

Observation of the immobilized antibody inside the pores

As shown in Figure 2c, the distribution of immobilized antibody in the membrane pores was directly observed using fluorescence spectroscopy. In this experiment, FITC-labeled antibody was immobilized on the membrane following the abovementioned protocol. The membranes were soaked in pure water and then frozen. Then, cross-sectional slices were cut (Figure S5). The membrane cross-sections were observed using confocal laser scanning microscopy (Zeiss LSM 780).

IL-6 detection using the membrane-based sensor

IL-6 detection test was conducted via permeation of (1) analyte (the antigen solution), (2) biotinylated secondary antibody, (3) HRP-labeled avidin, and (4) substrate (3,3',5,5'-tetramethyl benzidene, TMB) solution using the equipment shown in Figure S6. Based on our calculation,[3] the third step considerably contributes to the signal performance. Hence, to investigate the suitable permeation and concentration conditions, the background signals (the absorbance obtained when the abovementioned steps (1)–(4) were performed with [Antigen] = 0 pg/ml) were measured experimentally at different secondary antibody concentrations. Figure S7a shows that the background signal can be suppressed until 200 ng/ml of secondary antibody. Subsequently, the permeation time was also considered. In this test, the permeation time of the secondary antibody solution was varied from 10 to 40 min with a concentration of 200 ng/ml and flow rate of 50 µl/min. Figure S7b shows the background signal at each flow condition, and the background signal was suppressed until 30 min of flow. Therefore, in this study, we adopted the conditions of a 200-ng/ml concentration and a 30-min flow duration in the third step to obtain high signal and low background.

ELISA for IL-6 detection

A solution with 2.00 µg/mL of capture antibody was prepared by diluting anti-IL-6 antibody in phosphate-buffered solution (PBS). To coat the capture antibody on a 96-well plate, 100 µL of this solution per well was dropped and incubated overnight at room temperature. After rinsing each well three times using wash buffer (PBS-T, 0.05%), blocking was done with 300 µL of BSA solution (1 wt%) and the plate was incubated at room temperature for 1 h. After washing again, 100 µL of IL-6 solution diluted using PBS containing BSA (1 wt%) was added to each well and incubated for 2 h. After removing the remaining IL-6, 100 µL of biotinylated anti-IL-6 solution (50.0 ng/mL) diluted with PBS containing BSA (1 wt%) was added to well and incubated for 2 h and washed. Then, 100 µL of the streptavidin-HRP solution was added and incubated for 20 min. Subsequently, 100 µL of TMB solution was added and incubated for 15 min at room temperature. The absorbance was then measured using a microplate reader.

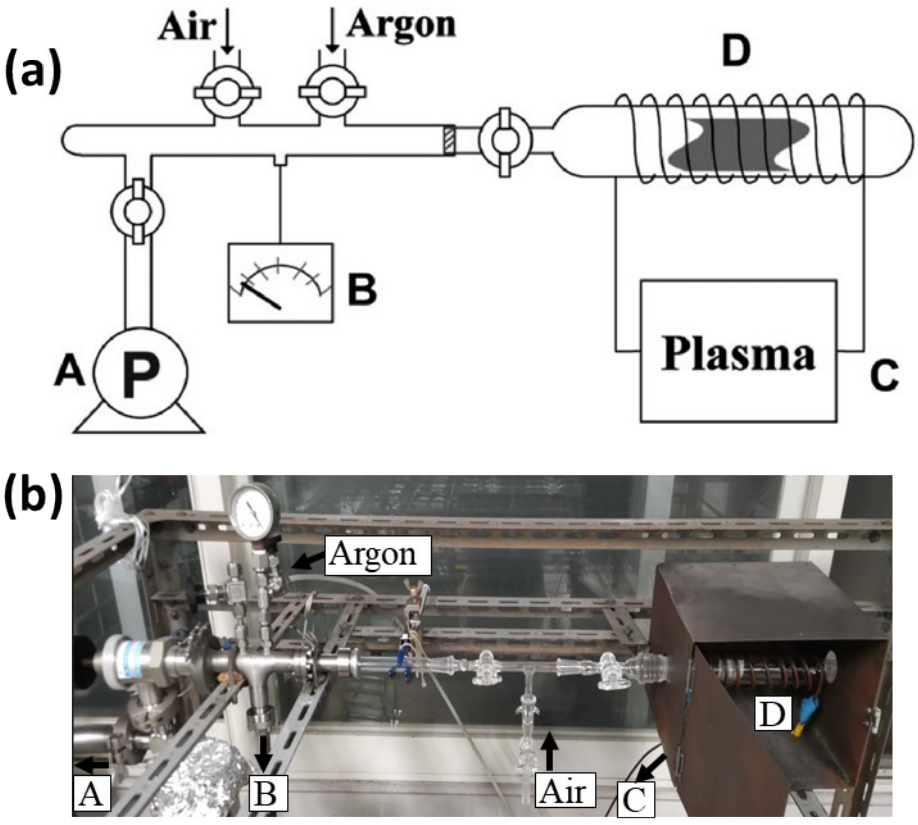

Figure S1. (a) Illustration and (b) Photograph of the equipment used for plasma treatment: A) vacuum pump, B) vacuum gage, C) RF generator, and D) glass tube containing membrane. RF, radiofrequency.

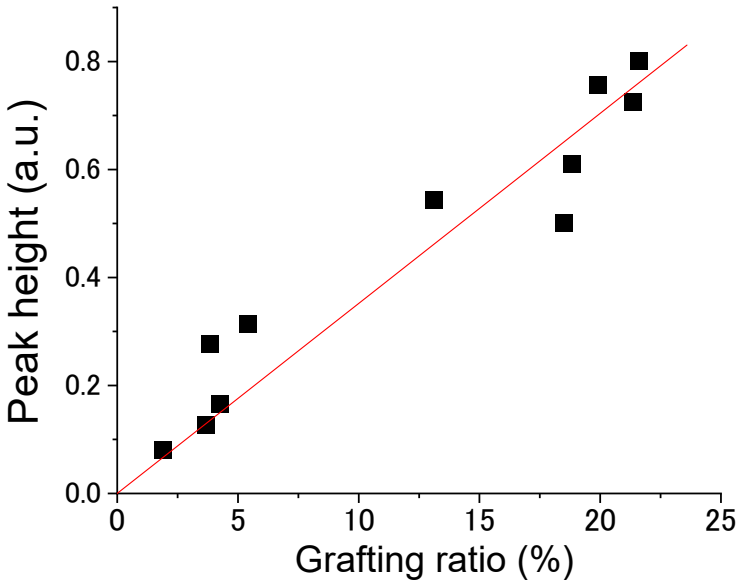

Figure S2. The relationship between the grafting ratio (%), which is estimated from the membrane weight change, and the height of the peak derived from graft polymer (at 1,730 cm^{-1}) in FT-IR.

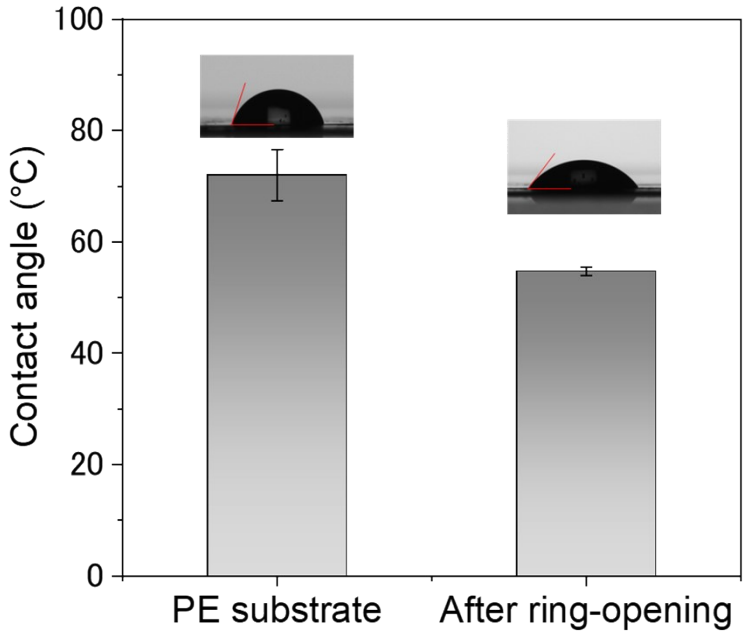

Figure S3. Water contact angle of PE substrate and membrane after ring-opening reaction. Measurements were performed at three different positions. Images of water droplets are also inserted in the figure.

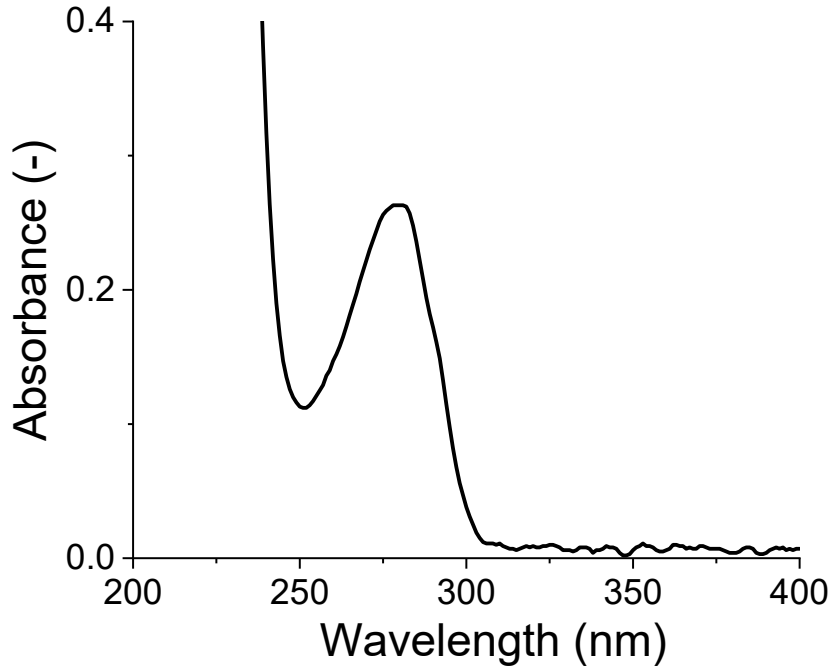

Figure S4. UV–vis spectrum of cyclooctyne-labeled capture antibody solution. The peak observed at 280 nm is derived from concentrated antibody.

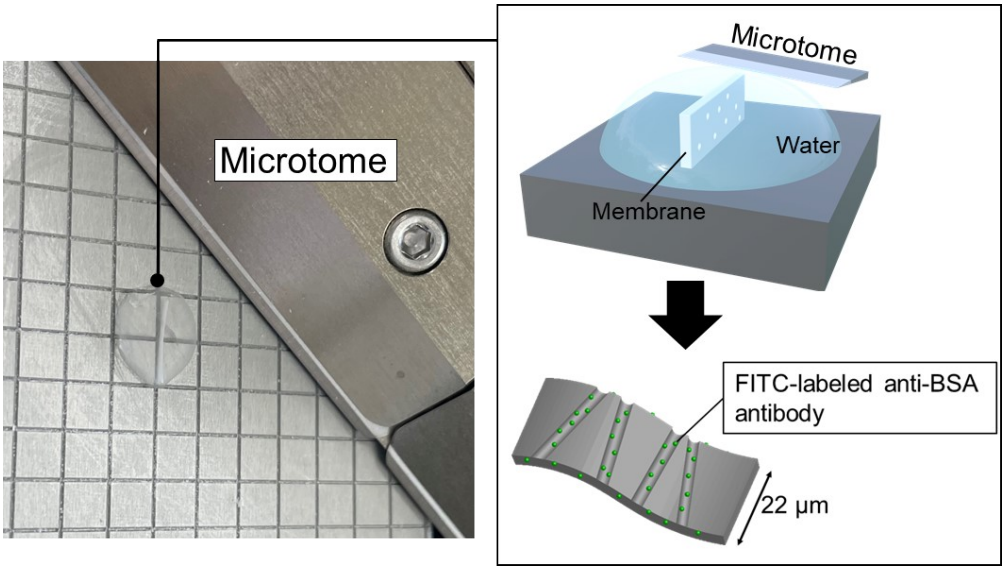

Figure S5. Preparation of the sample to observe the cross-sectional image of the membrane with FITC-labeled antibody.

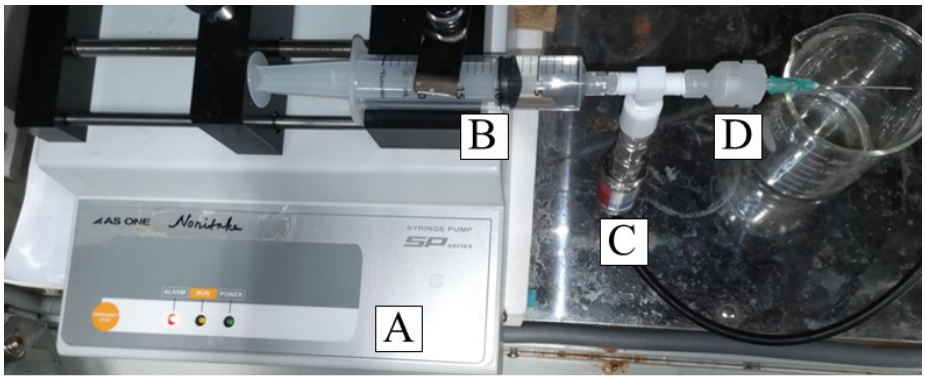

Figure S6. Permeation equipment used in the molecular recognition test: A) syringe pump, B) syringe containing pure water or sample solution, C) pressure sensor, and D) membrane holder containing the gating membrane. The effective area of the membrane holder is a circle with a diameter of 1 cm (0.79 cm^2).

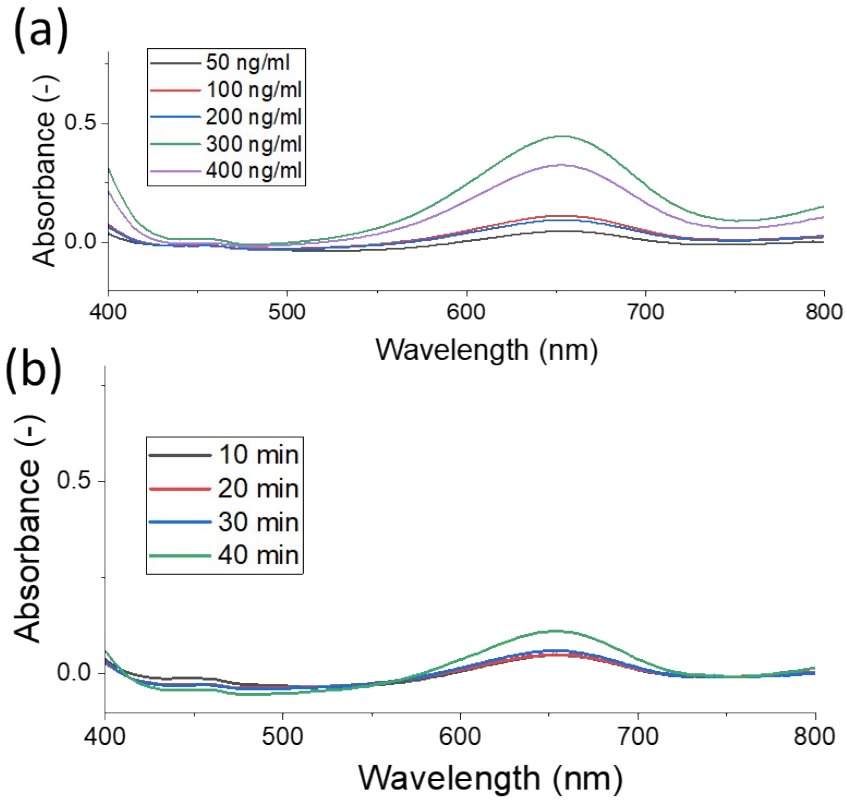

Figure S7. Improvement in the secondary antibody permeation step conditions. (a) Background signals with different concentrations of secondary antibody (flow rate = 50 µl/min and flow time = 10 min). (b) Background signals with different flow times (concentration = 200 ng/ml and flow time = 10 min).

References

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2. T. Yamaguchi, S. Nakao and S. Kimura, *Macromolecules*, 1991, **24**, 5522-5527.
3. H. Okuyama, T. Tamaki, Y. Oshiba, H. Ueda and T. Yamaguchi, *Analytical Chemistry*, 2021, **93**, 7210-7219.