Electronic Supplementary Information

In-situ Fabrication of Fully Negatively-charged Layer-by-layer Nanofilms on a Living Cell Surface

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Experimental Section

1. Materials

Poly (acrylic acid) (PAA, 25 kDa) hydrochloric acid (HCl, 5M), sulfuric acid (H_2SO_4, 95.0%), and Paraformaldehyde (PFA) were purchased from Wako Pure Chemical Industries. 2-[2-(2-Azidoethoxy)ethoxy] ethanamine (azide-EG_2-amine, 95%), and Cy5-amine were purchased from BroadPharm. Dibenzocyclooctyne-amine (DBCO-amine, >95%) was purchased from TCI. 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methyl morpholinium chloride (DMTMM, 97.5%) was purchased from Watanabe. Fluorescein glycine amide (FGA) was purchased from Funakoshi. Phalloidin-iFlor 555 was purchased from Abcam. Hoechst33342 was purchased from Thermo Fisher Scientific. 1x PBS 7.4 (PBS 7.4) was prepared by dissolving PBS powder (D-PBS without Ca and Mg, Powder from Nacalai tesque, INC.) to Milli-Q water.

2. Synthesis of PAA-EG_2-Azi

As describe previously,[1] PAA functionalized with various contents of azide groups as synthesized via DMTMM-mediated coupling of carboxyl groups of PAA with azide-EG_2-amine. 60 mg (0.83 mmol) of PAA as dissolved in 12 mL of sodium borate buffer (pH 8.5, 0.5 M). 460.8 mg (1.66 mmol) of DMTMM was added to the solution together with 36.3 mg (0.21 mmol) of azide-EG_2-amine to produce PAA-EG_2-Azi with 25 % azide feeding ratio. The solution was stirred at room temperature for 24 hours, then dialyzed against Milli-Q water and freeze-dried to get the final product.

3. Synthesis of PAA-DBCO

DBCO was conjugated to PAA via DMTMM-mediated coupling reaction. PAA (50 mg, 0.69 mmol) was mixed with DMTMM (192 mg. 0.69 mmol) in 13.33 ml of carbonate buffer solution (pH 8.5, 0,1 M) for 10 min. DBCO-amine (19.2 mg, 0.069 mmol) dissolved in 3.33 mL of THF. Then add to the mixture solution dropwise. The reaction kept stirring at room temperature for 24 hours. The product was purified via dialysis (MWCO: 8000) against Milli-Q water, methanol, and Milli-Q water for 1 day each.

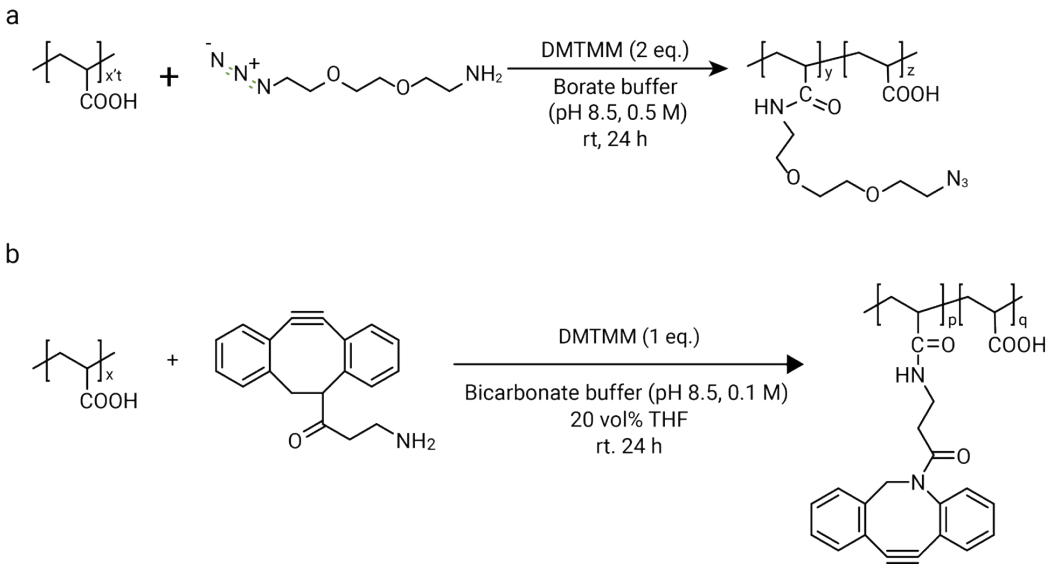

Figure S1 Synthesis of PAA-EG_2-Azi (a) and PAA-DBCO (b)

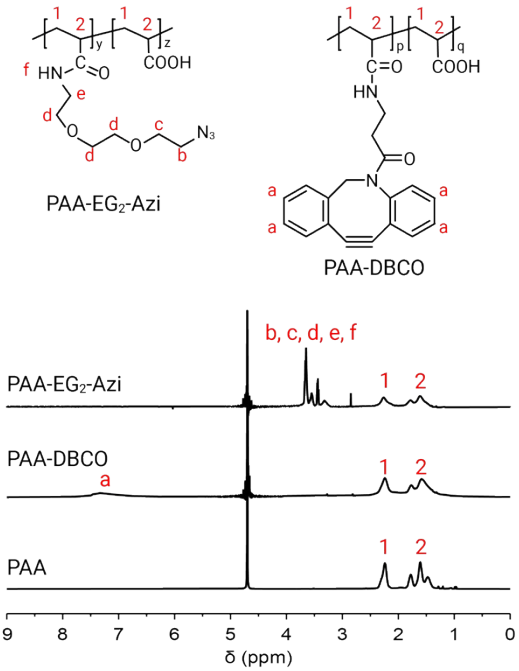

Figure S2 ^{1}H-NMR (400 MHz, D_2O, 25 °C) spectra of PAA-EG_2-Azi, PAA-DBCO,

and PAA

4. Click reaction monitoring using UV-vis absorbance.

PAA-EG_2-Azi and PAA-DBCO were dissolved in 1X PBS to obtain the polymer solution with the concentration of 3.2 mg/mL and 0.32 mg/mL respectively. 1.5 mL of PAA-EG_2-Azi and PAA-DBCO solutions were mixed in a cuvette, then pipetting 5 times. After mixture, the concentration of PAA-EG2-Azi and PAA-DBCO changed to 1.6 mg/mL and 0.16 mg/mL. the UV-Vis spectra were measured at a certain time interval.

5. Preparation and characteristics of Cu-free fully negative-charged layer-by-layer (LbL) nanofilm

The quantitative measurement of the LbL assembly was analyzed using a quartz crystal microbalance (QCM, AFFINIX Q8, ULVAC) as our previously reported protocol.[1] Briefly, QCM sensors (QCM01S, ULVAC) were treated with piranha solution (H_2SO_4:35% H_2O_2 = 3:1) for 10 min and repeated 3 times. 100 µL of PBS 7.4 was added to the cells until the frequency was stable. 5 µL of the concentrated PAA-EG_2-Azi (3.36 mg/mL, PBS 7.4) solutions was added to the cells and diluted to the desired concentration (0.16 mg/mL). After 15 min of deposition, PAA-EG_2-Azi solutions were removed, and the sensors were rinsed with PBS 3 times. Then the frequency was stabilized in new PBS, 5 µL of the concentrated PAA-DBCO (3.36 mg/mL, PBS 7.4) solutions was added into the cell s for the second layer assembly. By alternating switch the polymer solutions, nanofilms were assembled on the QCM sensors. The frequency shift (ΔF) was recorded, and the deposited mass (Δm) was calculated based on the Sauerbrey equation[2]:

$$-\Delta m(ng/cm^2) = 0.62\Delta F(Hz) \#(S1)$$

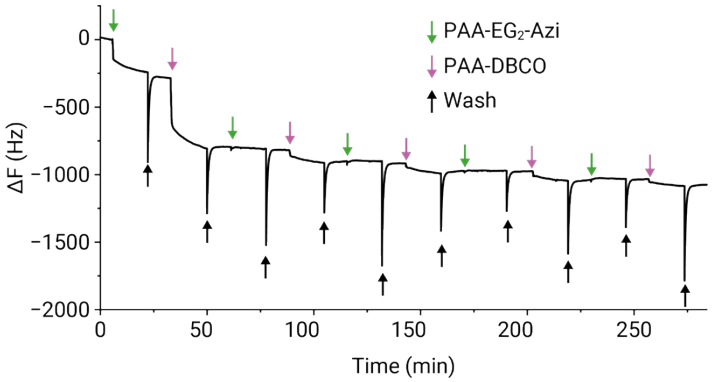

Figure S3 Frequency shifts (ΔF) plotted against time for a (PAA-EG_2-Azi/PAA-

DBCO)$_5$ LbL nanofilm assembly with a polymer concentration of 0.16 mg/mL. Vertical arrows indicate the moment of adding polymer solutions to the QCM cell and the start of rinsing steps using PBS 7.4.

The pH-responsive measurement and stability evaluation of LbL nanofilms were accessed in situ by QCM. After film fabrication, they were incubated in 100 μL PBS 7.4 or PBS 6.5.

6. AFM observation

For the AFM (Shimadzu, SPM-Nanoa) measurement, layer-by-layer nanofilms were fabricated on silicon wafers (As one, 2-960-05). (AFM measurement mode: Dynamic (lq); probe: OMCL-AC200TS-C3)

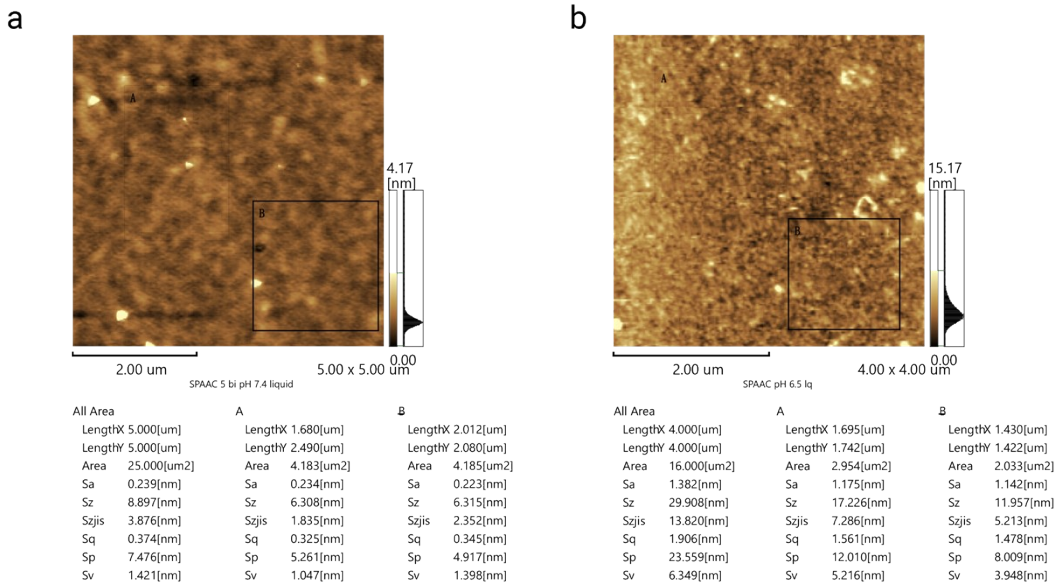

Figure S4 AFM surface morphology and surface roughness for (PAA-EG$_2$-Azi/PAA-DBCO)$_5$ LbL nanofilms in PBS 7.4 (a) and PBS 6.5 (b).

7. Synthesis of FGA modified PAA-EG$_2$-Azi

PAA-EG$_2$-Azi (20 mg) was dissolved in 5 mL of carbonate buffer solution (pH 8.5, 0.1 M). DMTMM (116.9 mg) was added to activate carboxyl groups for 10 min. FGA (0.58 mg) was dissolved in DMSO (0.5 mL), then added dropwise to the PAA-EG$_2$-Azi solution. The mixture was kept stirring in an ice bath for 1 h then reacted away from light in room temperature for 24 h. The product was purified via dialysis (MWCO: 8000) in the dark against Milli-Q water for 3 days followed by freeze-drying.

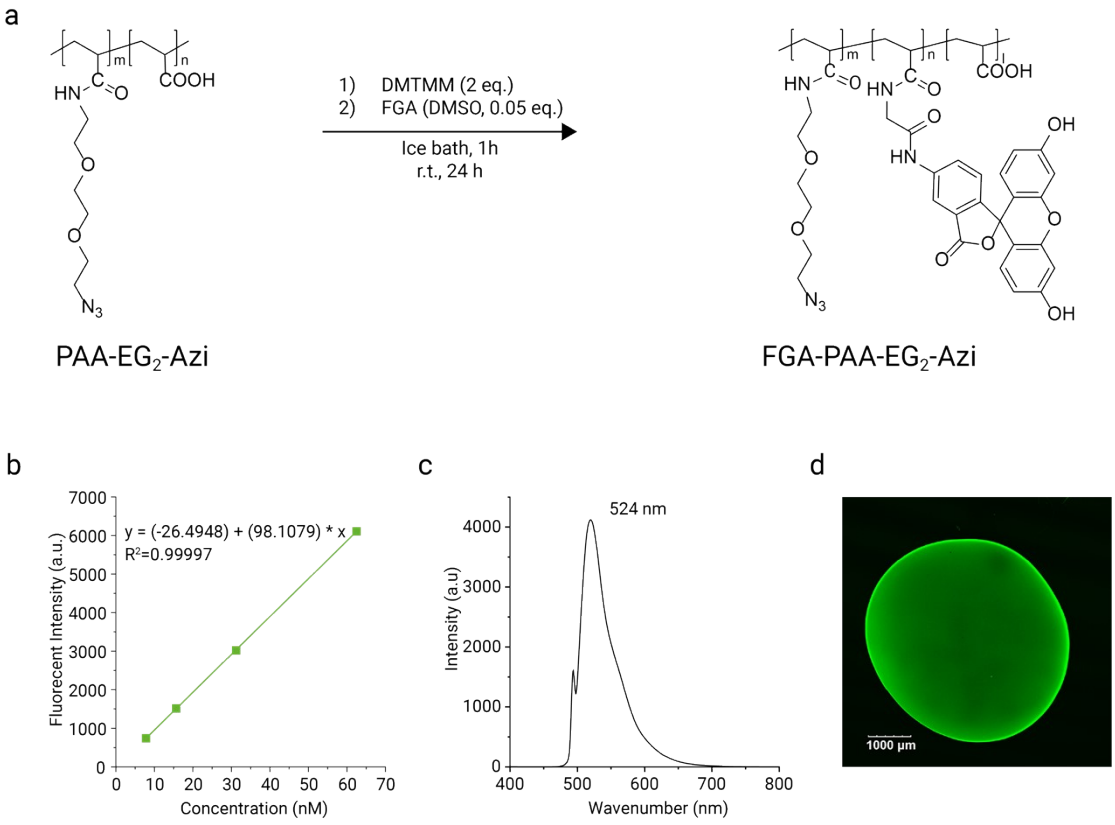

Figure S5 (a) Synthesis of FGA labeled PAA-EG_2-Azi (b) Standard curves of FGA (c) Fluorescent spectrum of FGA-PAA-EG_2-Azi with the concentration of 0.2 mg/mL. (d) CLSM image of FGA-PAA-EG_2-Azi solution droplet (1 mg/mL)

8. Synthesis of Cy5 modified PAA-DBCO

PAA-DBCO (20 mg) was dissolved in 5 mL of carbonate buffer solution (pH 8.5, 0.1 M). DMTMM (105.7 mg) was added to the polymer solution and stirred for 10 min. Cy5-amine (0.53 mg) was dissolved in DMSO (0.5 mL), then added dropwise to PAA-DBCO solution. The solution was kept stirring in an ice bath for 1 h then reacted in the dark at room temperature for 24 h. The product was purified by dialysis (MWCO) in the dark against Milli-Q water for 3 days.

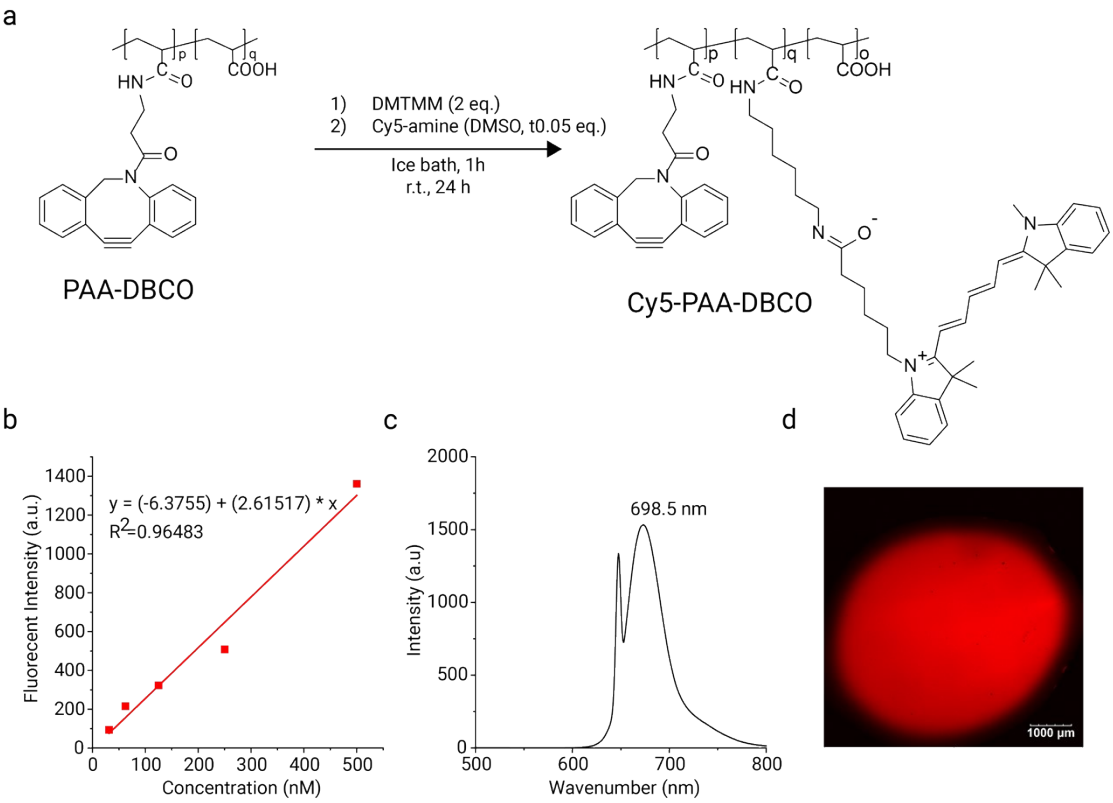

Figure S6 (a) Synthesis of Cy5 labeled PAA-DBCO (b) Standard curves of Cy5-amine (c) Fluorescent spectrum of Cy5-PAA-DBCO with the concentration of 0.25 mg/mL. (d) CLSM image of Cy5-PAA-DBCO solution droplet (1 mg/mL)

9. Fabrication of LbL nanofilms using fluorescent labeled polymers on QCM sensors.

The fabrication of LbL nanofilms using fluorescent labeled polymers was also monitored using QCM as the non-fluorescent labeled polymers. Briefly, QCM sensors (QCM01S, ULVAC) were treated with piranha solution (H_2SO_4:35% H_2O_2 = 3:1) for 10 min and repeated 3 times. 100 μL of PBS 7.4 was added to the cells until the frequency was stable. 5 μL of the concentrated FGA-PAA-EG_2-Azi (3.36 mg/mL, PBS 7.4) solutions was added to the cells and diluted to the desired concentration (0.16 mg/mL). After 15 min of deposition, FGA-PAA-EG_2-Azi solutions were removed, and the sensors were rinsed with PBS 3 times. Then the frequency was stabilized in new PBS, 5 μL of the concentrated Cy5-PAA-DBCO (3.36 mg/mL, PBS 7.4) solutions was added into the cell s for the second layer assembly. By alternating switch the polymer solutions, nanofilms were assembled on the QCM sensors. The frequency shift (ΔF) was recorded, and the deposited mass (Δm) was calculated based on the Sauerbrey equation[2]:

$$-\Delta m(ng/cm^2) = 0.62\Delta F(Hz)\#(S1)$$

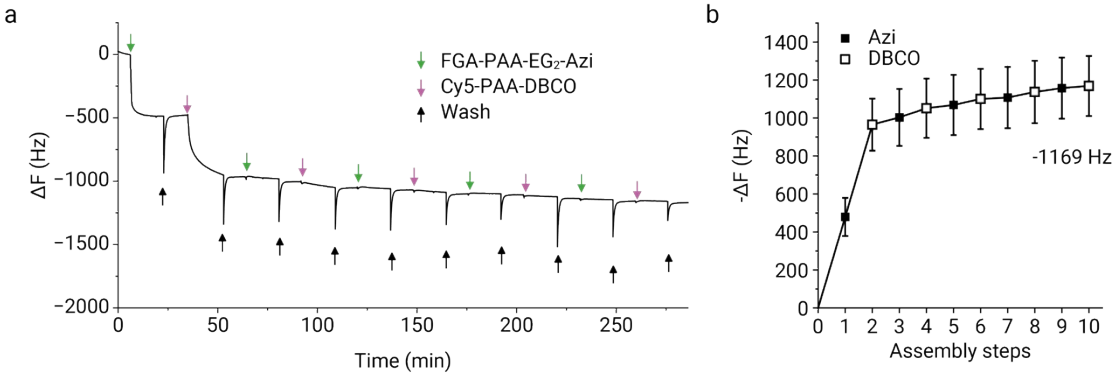

Figure S7 (a) Frequency shifts (ΔF) plotted against time for a (FGA-PAA-EG_2-Azi/Cy5-PAA-DBCO)$_5$ LbL nanofilm assembly with a polymer concentration of 0.16 mg/mL. Vertical arrows indicate the moment of adding polymer solutions to the QCM cell and the start of rinsing steps using PBS 7.4. (b) Frequency shifts (ΔF) of (FGA-PAA-EG_2-Azi/Cy5-PAA-DBCO)$_5$ LbL nanofilm at each step. The polymer concentration is 0.16 mg/mL.

10. Fabrication of LbL nanofilms using fluorescent labeled polymers on glass substrates.

Micro slid glass (Matsunami, S2441) was cut into stripes with 0.5 × 2.6 cm size. Polymers were dissolved in Milli-Q water with the concentration of 0.16 mg/mL in PBS 7.4. Glass stripes were cleaned with Piranha solution for 30 min, then rinsed with Milli-Q water and dried with nitrogen flow. Glass strips were alternating immersed in FGA-PAA-EG_2-Azi and Cy5-PAA-DBCO solutions for 15 min, with PBS 7.4 rinsing in between. After wanted layers numbers, the samples were observed using FV3000 with same excitation strength. The fluorescent intensity was measured using ImageJ.

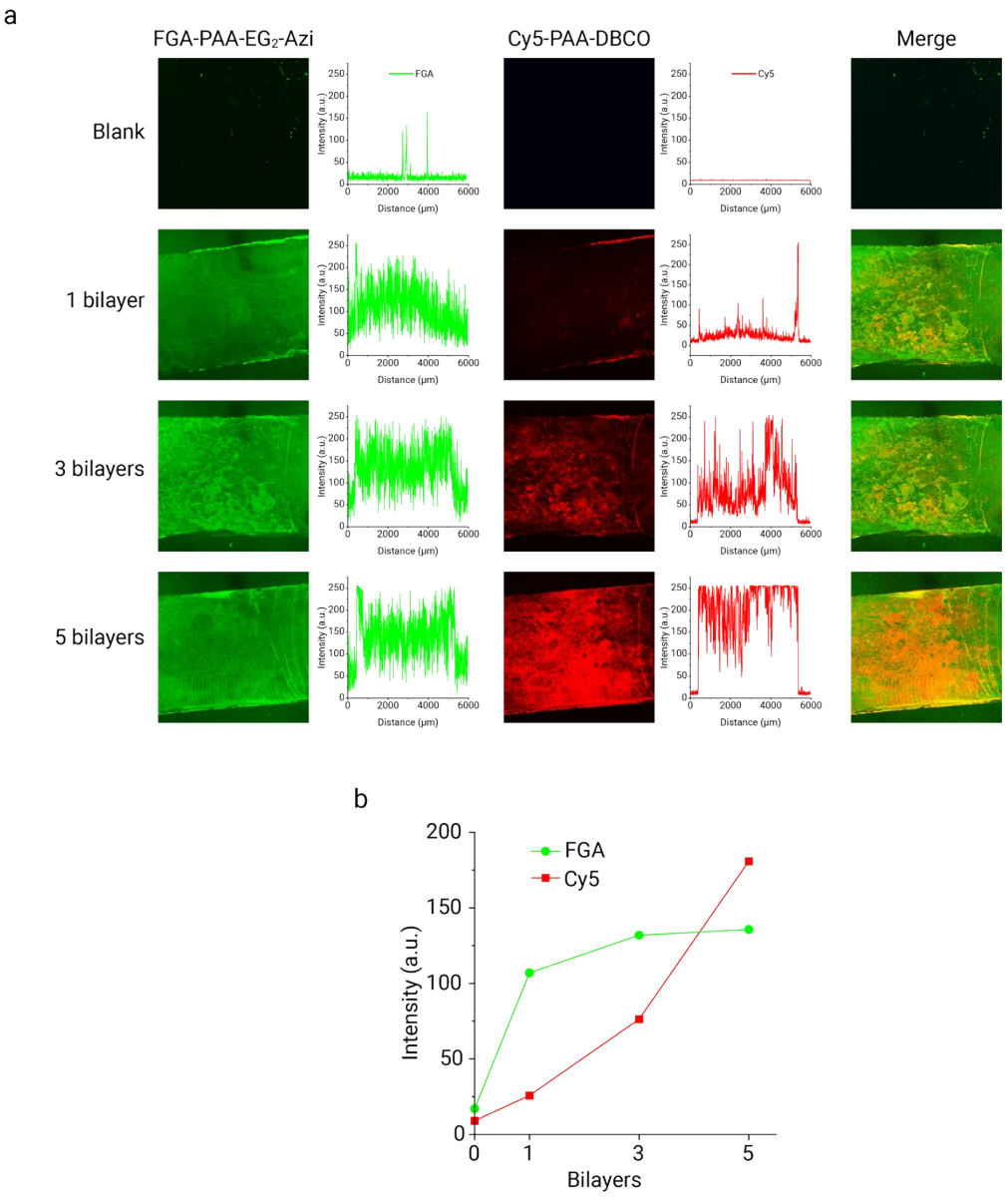

Figure S8 (a) CLSM images of $(FGA\text{-}PAA\text{-}EG_2\text{-}Azi/Cy5\text{-}PAA\text{-}DBCO)_n$ LbL nanofilm assembled on glass substrates. (b) Fluorescent intensity of CLSM images with different bilayer numbers. CLSM Images were taken with the same laser power.

11. Cell culture

NHDF (passage 9-11) were cultured with DMEM containing 10% FBS and 1% penicillin/streptomycin in 5 % CO_2 at 37 °C.

12. Cell viability

The viability of NHDF with Cu-free nanofilms was performed using a Live/Dead viability assay kit and WST-8 kit assay. For Live/Dead viability assay kit, 1.0×10^5 NHDF were seeded in each well (24-well plate) and then incubated at 37 °C for 24 h.

LbL nanofilms were assembled on the cell surface and incubated for another 24 h. After further washing with PBS, 300 μL of **PBS** solution containing Calcein AM (1 μM) and EthD-1 (2 μM) was added into each well, followed by incubation at 37 °C for 45 min in the dark. After incubation, each well was rinsed with PBS 3 times. Images were obtained using a confocal laser scanning microscope.

For WST-8 kit assay, 100 μL of 1.0×10^4 NHDF were cultured in 96-well plate at 37 °C for 24 h. Then nanofilms were assembled on the cell surface and incubated for another 24 h. After incubation, the cells were washed with PBS 3 times, 100 μL of cell count reagent SF/DMEM (1:9) was added to each well. The cells were incubated for another 3 h at 37 °C. And then transferring the supernatant to a new well, the absorbance of supernatant at 450 was measured by microplate reader. The cell numbers after LbL were counted by trypan blue staining. The normalized cell viability was calculated.

13. In-situ layer-by-layer on cell surface

LbL nanofilms were assembled on the top of fibroblasts. To distinguish the cells and nanofilms. 2 mL of 2×10^6 NHDF was incubated in a 35 mm glass-bottom dish for 24 h. After washing with PBS 3 times, 2 mL FGA-PAA-EG_2-Azi and Cy5-PAA-DBCO with the concentration of 0.16 mg/mL were deposited alternately on the NHDF surface for 5 bilayers and incubated in 2 mL DMEM (10% FBS, 1% anti) at 37 °C for 24 h. Cells and nanofilms were observed with confocal laser scanning microscope (FluoView FV3000). The results were analyzed by Imaris and ImageJ.

14. Actin Staining

After LbL on the NHDF surface and incubated for 24 h, samples were washed with PBS 3 times and fixed by 4% paraformaldehyde (PFA) for 15 min at room temperature. After washing with PBS 3 times, the samples were incubated with Phalloidin-iFlor 555 in 1% BSA/PBS solution at room temperature for 2 h. The nuclei were counterstained with Hoechst33342. After washing with PBS 3 times, the samples were observed with a confocal laser scanning microscope.

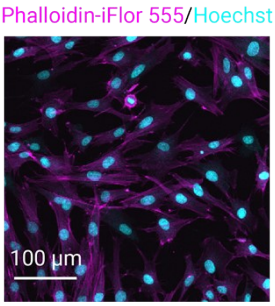

Figure S9 CLSM images of the cell without depositing LbL nanofilms.

15. Safranin O (SO) absorption

NHDF coated with $(PAA\text{-}EG_2\text{-}Azi/PAA\text{-}DBCO)_5$ (10 layers), $PAA\text{-}EG_2\text{-}Azi$ (1 layer), and without nanofilms (Control) were cultured. Samples were incubated in 10 μg/mL of Safranin O (SO)/PBS for 30 min in the dark. After incubation, the fluorescent intensities of supernatants were measured. The cell samples were washed with PBS 3 times then observed by FV3000. The linear scanning of the fluorescence intensity was measured using ImageJ.

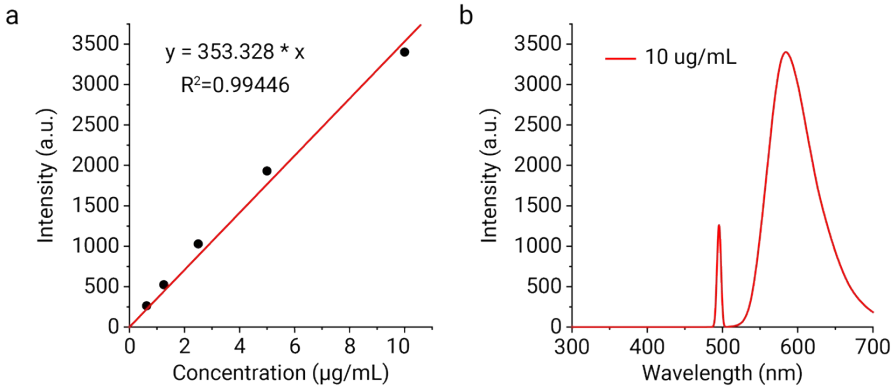

Figure S10 Standard curves (a) and fluorescent spectrum of Safranin O (b).

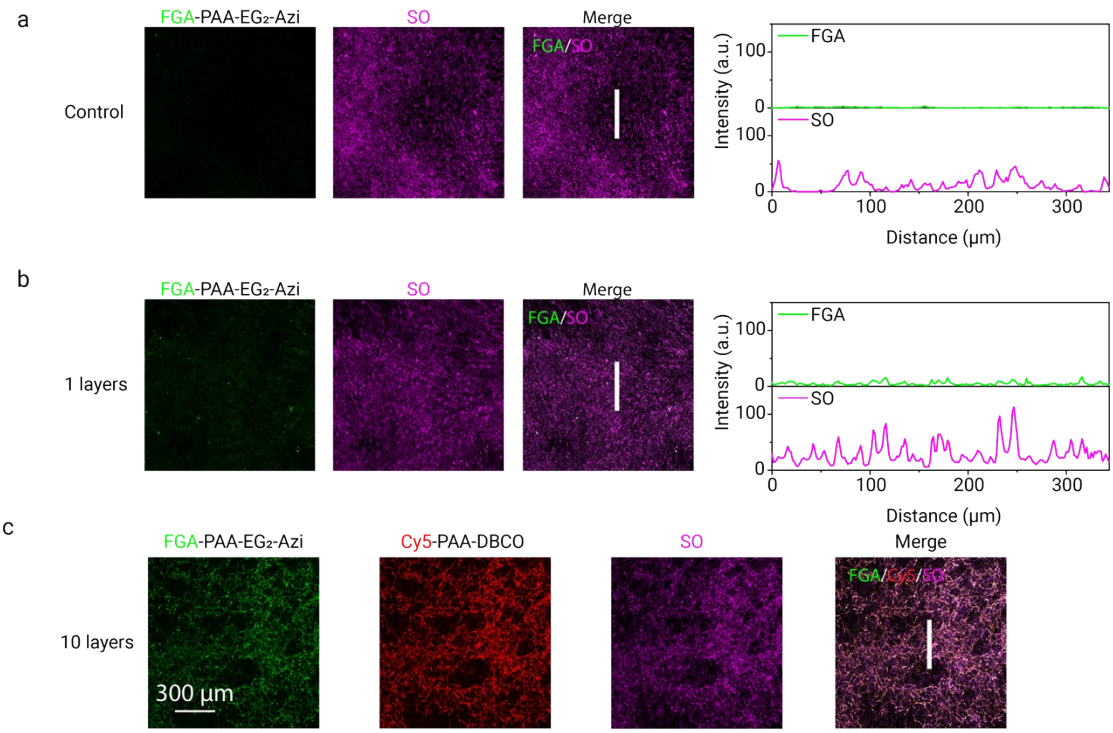

Figure S11 (a-b) Left: low magnification CLSM images of cells without nanofilms (a) and with 1 layer of PAA-EG_2-Azi_5 (b); Right: Linear scanning of the fluorescence intensity profiles along the white line. (c) Low magnification CLSM images of cells with $(PAA\text{-}EG_2\text{-}Azi/PAA\text{-}DBCO)_5$ obtained after 30 min incubation in the solutions of SO (10 μg/mL). (Green: FGA, red: Cy5, violet: SO).

16. Characterization methods and statistic

The chemical structure of the functionalized polymers was analyzed by ^{1}H-NMR (Jeol, JNM-GSX 400, Japan). Fluorescence images were observed with confocal laser scanning microscopy (CLSM, FV300, Olympus, Japan). The AFM observing cells were conducted using 3D mapping in PBS 7.4 (Probe: CONT, Nanoworld).

17. Statistical analysis

All values are presented as means ± standard deviation (SD). Statistical analysis of the data was performed with Student's *t*-test or One-way *ANOVA*.