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Supplementary Information

Surfaces immobilized with oligo-prolines prevent protein adsorption and cell adhesion

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1. Experimental methods

1. 1. Synthesis of oligo-prolines

Oligo-prolines, such as Ac-Cys-$(Pro)_6$-$CONH_2$ (Pro6) and Ac-Cys-$(Pro)_9$-$CONH_2$ (Pro9), were synthesized by the typical Fmoc solid-phase procedure on the semi-automatic peptide synthesizer (ResPep SLi; INTAVIS Bioanalytical Instruments AG, Germany). Briefly, the C-terminal Pro residue was attached to the NH-SAL-PEG resin (0.22 mmol/g, 90 μm) using Fmoc-L-Pro-OH with 1-hydroxy-1H-benzotriazole hydrate (HOBt) and N-methylmorpholine (NMM) in dimethylformamide (DMF). Subsequently, the Fmoc group was removed using 20% (v/v) piperidine in DMF. Fmoc-protected amino acids, Fmoc-L-Pro-OH, and Fmoc-L-Cys(Trt)-OH were attached for the elongation of the peptide chain with HOBt/NMM. The N-terminus of the peptide was protected with the acetyl group using acetic anhydride and the cleavage of oligo-prolines was implemented using trifluoroacetic acid (TFA) containing triisopropylsilane, thioanisole, ethanedithiol, and water (82.5:5:5:2.5:5 (v/v)). After 2 hours of the cleavage reaction, oligo-prolines were precipitated and purified in tenfold amount of diethyl ether. All reagents for peptide synthesis were purchased from Watanabe Chemical Industries (Hiroshima, Japan). The purity of oligo-prolines was determined by the HPLC system (Shimadzu, Kyoto, Japan) with a reversed-phase column (YMC-Pack PROTEIN-RP, 4.6×250 mm; YMC CO., LTD., Kyoto, Japan). The HPLC system was operated by a mobile phase of water (0.1% (v/v) TFA) and acetonitrile (0.1% (v/v) TFA) with a linear gradient. In addition, oligo-prolines were characterized using MALDI-TOF/MS (Microflex LT; Bruker Daltonics, Bremen, Germany) with alpha-Cyano-4-hydroxycinnamic acid as a matrix.

1. 2. Circular dichroism (CD)

CD spectra were measured by J-1100 spectropolarimeter (Jasco Co., Tokyo, Japan) with a standard analysis program. The temperature was controlled at 37°C using the Peltier-type cell holder, and spectra were recorded with a 0.1 cm for the cell path length, a scanning speed of 10 nm/min, and a spectral bandwidth of 1.0 nm in the wavelength range from 190 to 260 nm. Oligo-prolines were dissolved in water at 0.1 mM. Data are represented in residue molar ellipticities ([θ] deg cm^2 $dmol^{-1}$).

1. 3. Preparation of oligo-proline immobilized surface

Glass cover slips (13 mm diameter, 0.17-0.25 μm thickness; Matsunami Glass Ind., Ltd., Osaka, Japan) were used as a substrate for the fabrication of the gold (Au)-sputtered surface. Glass cover slips were cleaned in piranha solution (H_2SO_4/H_2O_2=5:1 (v/v)) for 30 minutes and rinsed in ultra-pure water. After drying in air, glass cover slips were treated using the UV/O_3 chamber (ProCleaner™: BioForce Nanosciences, Inc., IA, USA) for 20 minutes. The sputtering was

accomplished on radio frequency (RF) magnetron sputtering system (Kenix Co. Ltd., Hyougo, Japan). The Cr layer was initially deposited on the glass cover slips as an adhesion layer using a chromium target (purity 99.9%, supplied by Osaka Asahi Co., Ltd., Osaka, Japan). Subsequently, the Au layer was deposited using a gold target (purity 99.9%, supplied by Osaka Asahi Co., Ltd., Osaka, Japan). After UV/O_3 treatment, Au-sputtered glass cover slips were immersed in an aqueous solution of the oligo-prolines (1.0 mM) for 2 hours at room temperature. Oligo-proline immobilized substrates (Pro6-s and Pro9-s) were rinsed in ultra-pure water and dried in vacuo before subsequent experiments. In addition, Au-sputtered glass cover slips were immersed in an aqueous solution of 3-aminopropanethiol (MPAC; Sigma-Aldrich, St. Louis, MO, USA) and aminated surfaces (MPAC-s) were obtained as controls.

1. 4. Characterization of oligo-proline immobilized surface

In order to determine the molecular density, oligo-prolines were immobilized on a gold-coated quartz crystal sensor and the resonance frequency was monitored by a quartz crystal microbalance with a dissipation (QCM-D) instrument (Q-Sense E1; Biolin Scientific,
Gothenburg, Sweden). Briefly, a gold-coated quartz crystal sensor was mounted in the flow chamber and equilibrated in ultra-pure water for 10 minutes at a constant temperature of 37◦C and a flow rate of 100 μL/min. An aqueous solution of oligo-prolines (1.0 mM) or MPAC (1.0 mM) was injected to the flow chamber and maintained at a constant flow rate of 100 μL/min for 30 minutes, and then rinsed with ultra-pure water until the resonance frequency and energy dissipation stabilize. Changes in the resonance frequency (Δf) before and after molecular immobilization were calculated, and the mass of the immobilized oligo-prolines or MPAC per unit area (m) was converted from Δf using Sauerbrey's equation:

$$\mathrm{m} = -\frac{C}{n}\Delta f$$

where C (= 17.7 ng cm^{-2} Hz^{-1} at f =5 MHz crystals) is the sensitivity constant and n (=1, 3,...) is the overtone number.

The water wettability of the oligo-proline immobilized surfaces was evaluated by the static water contact angle. Samples were pre-hydrated by immersion in ultra-pure water for 60 minutes. The water CA of oligo-proline immobilized surfaces was measured by employing the sessile drop method in air using a goniometer (CA-XP; Kyowa Interface Science Co., Tokyo, Japan). The volume of water droplets was 5 μL. Photographs of the water droplets were taken after 30 s, and the CAs were measured.

1.5. Adsorption of proteins and serum components on oligo-proline immobilized surfaces

Protein adsorption was subsequently measured using a crystal sensor with QCM-D instrument

after molecular immobilization. The crystal sensor was equilibrated in 0.1 M of phosphate buffer solution (PBS) (0.1 M, pH 7.4) for 10 minutes at a constant temperature of 37◦C and a flow rate of 100 μL/min. Then, either of the protein solutions in PBS (human serum albumin (HSA; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan): 3.0% (w/w), human fibrinogen (HFG; FUJIFILM Wako Pure Chemical Corporation): 0.3 mg/ml, fetal bovine serum (EquaFETAL®; Atlas Biologicals, Inc., CO, USA): 10% (v/v)) was injected at a constant flow rate of 100 μL/min for 60 minutes, and then rinsed with PBS. The mass of the adsorbates per unit area was converted from Δf using the Sauerbrey's equation.

1. 6. Adhesion of fibroblasts on oligo-proline immobilized surfaces

In order to visualize F-actin in living cells, fibroblasts (NIH/3T3 clone 5611; JCRB Cell Bank, Osaka, Japan) were transfected with pCAG-LifeAct-TagRFP (ibidi, Martinsried, Germany) using Lipofectamine® 2000 (Invitrogen Corporation, CA, USA). Fibroblasts were cultivated in Dulbecco's modified eagle medium (DMEM) (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml and 100 μg/ml of streptomycin in a humidified incubator at 37℃ with 5% CO_2. Oligo-proline immobilized substrates were washed with DMEM once. Cultured fibroblasts were trypsinized, washed with DMEM, and counted using the cell counter (model R1; Olympus, Tokyo, Japan). The concentration of fibroblasts was adjusted to 1.0×10^6 cell/ml and a 500 μL of cell suspension was subsequently seeded on Pro6-s, Pro9-s, and MPAC-s using the culture medium. After 3 or 6 hours of incubation, samples were gently rinsed three times with the culture medium to remove unattached cells, and adherent cells were fixed in 10% (v/v) buffered formalin for 30 minutes. Samples were mounted on ProLong Gold with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen Corporation) and observed by confocal laser-scanning microscopy (Nikon C2; Nikon Inc., Tokyo, Japan) with the operating software (NIS-Elements C; Nikon Inc.). The number of adherent cells was counted as a total of five images that were randomly taken in a sample.

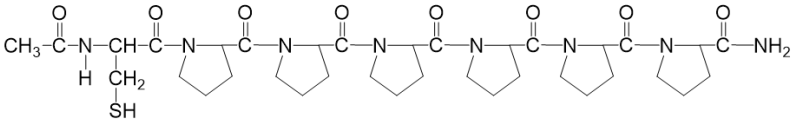
1. 7. Statistical analysis

Statistical analyses were examined using a one-way analysis of variance (ANOVA) with the Tukey-Kramer *post-hoc* test. Data are given as mean ± standard deviation (SD) (n = 3) and a *P* value smaller than 0.05 ($p \leq 0.05$) was regarded as statistically significant.

2. Characterization of P6 and P9

Oligo-prolines, Pro6, and Pro9 were synthesized by a Fmoc solid-phase procedure with no special protecting groups (Fig. S1 (A) and (B)). The purity of Pro6 and Pro9 were determined by a reverse-phase HPLC analysis and were 83.6% and 90.6%, respectively (Fig. S2 (A) and (B)). Pro6 and Pro9 were characterized using MALDI-TOF/MS (Microflex LT; Bruker Daltonics, Bremen, Germany) and the detected peaks completely matched the theoretical molecular weight (Fig. S3 (A) and (B)).

(A) Pro6 [Mw: 744.91]



(B) Pro9 [Mw: 1036.26]

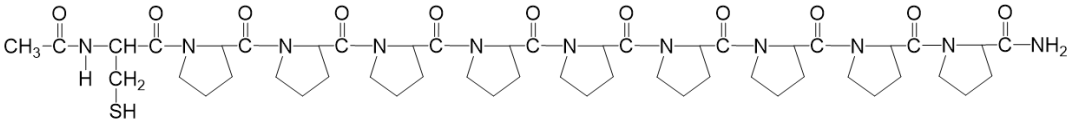


Fig. S1 Molecular structures of Pro6 and Pro9.

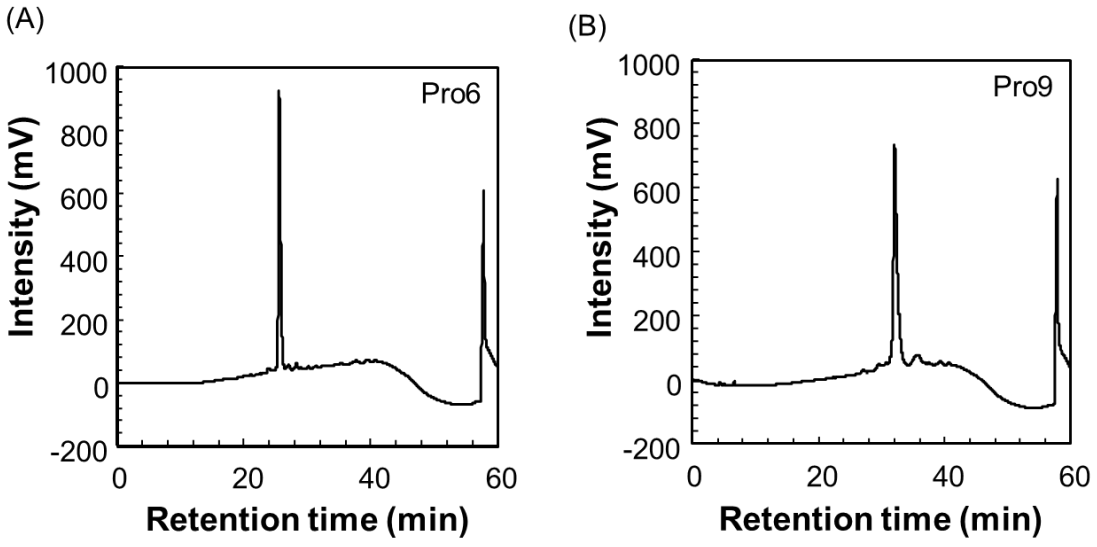


Fig. S2 Reversed-phase chromatograms of (A) Pro6 and (B) Pro9.

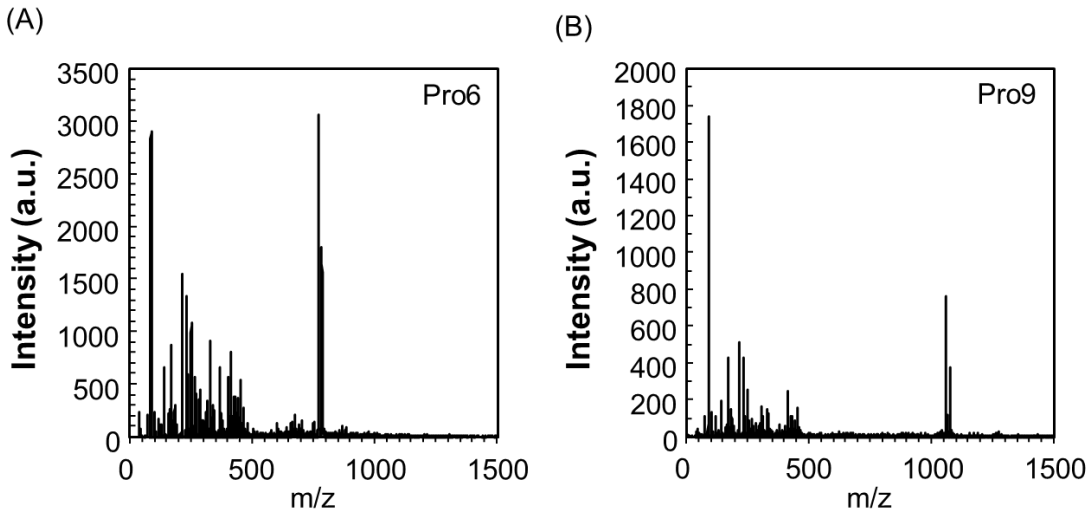


Fig. S3 MALDI-TOF/MS spectra of (A) Pro6 and (B) Pro9.

3. Analysis of proline immobilized surfaces by Fourier transform infrared reflection absorption spectroscopy

The molecular structure of oligo-prolines immobilized on Pro6-s and Pro9-s was analyzed by Fourier transform infrared reflection absorption spectroscopy (FT-IR/RAS) (FT/IR-4200; Jasco Co.) equipped with a reflector (RAS PRO410-H, Jasco Co.) and a mercury-cadmium telluride (MCT) detector cooled by liquid nitrogen. Au-sputtered glass cover slips were used as a reference substrate. On both Pro6-s and Pro9-s, the band at 1655-1660 cm^{-1} was found in FT-IR/RAS (Fig. S4). It has been reported that the bands at 1640–1650 cm^{-1} and 1651 cm^{-1} generally indicate water absorption and random coil structure, respectively (M. Lorusso et al., Soft Matter, 2011, 7, 6327–6336). FT-IR/RAS of Pro6-s and Pro9-s were measured using dried samples and the detected bands existed at a value higher than 1651 cm^{-1}. In addition, this band revealed a blue shift according to the increase in the number of proline residues. These results suggest that the bands detected at 1655-1660 cm^{-1} on Pro6-s and Pro9-s are indicative of a PP-II conformation that lacks intermolecular hydrogen.

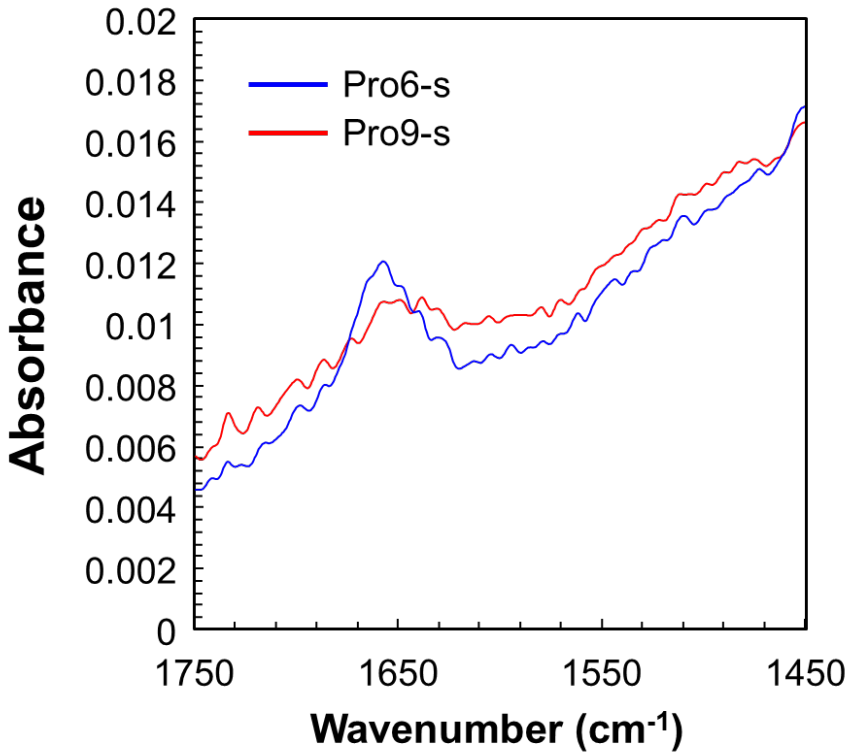


Fig. S4 FT-IR/RAS spectra of Pro6-s and Pro-9-s at Amide I and Amide II regions.

4. Cytotoxicity assay on oligo-proline immobilized surfaces

Fibroblasts (NIH/3T3 clone 5611) transfected with pCAG-LifeAct-TagRFP were seeded at a density of 2.5×10^4 cells/cm^2 in 96-well plates. Fibroblasts were cultivated in Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 100 U/ml and 100 µg/ml streptomycin in a humidified incubator at 37℃ with 5% CO_2. After 24 hours in the culture, the medium of the culture was changed, and the different concentrations of Pro6 or Pro9 were added to the wells. The fibroblasts were allowed in the culture for 5, 9, and 24 hours in the presence of Pro6 or Pro9. The relative toxicity of oligo-prolines on the cells was assessed with the WST-1 cell proliferation assay kit (Takara Bio Inc., Shiga, Japan). 10 µl of WST-1 solution were added to the media in each well and incubated for 1 h, and then the absorbance of the media was measured at 450 nm on a multimode microplate reader (SPARK10M; Tecan, Mannedorf, Switzerland). The cell viability was normalized to the viability of the fibroblasts cultured with no oligo-prolines. The cell viability was more than 90% for both Pro6 and Pro9 at all concentrations (Fig. S5 (A) and (B)). The results demonstrate that Pro6 and Pro9 possess no adverse effects on fibroblasts.

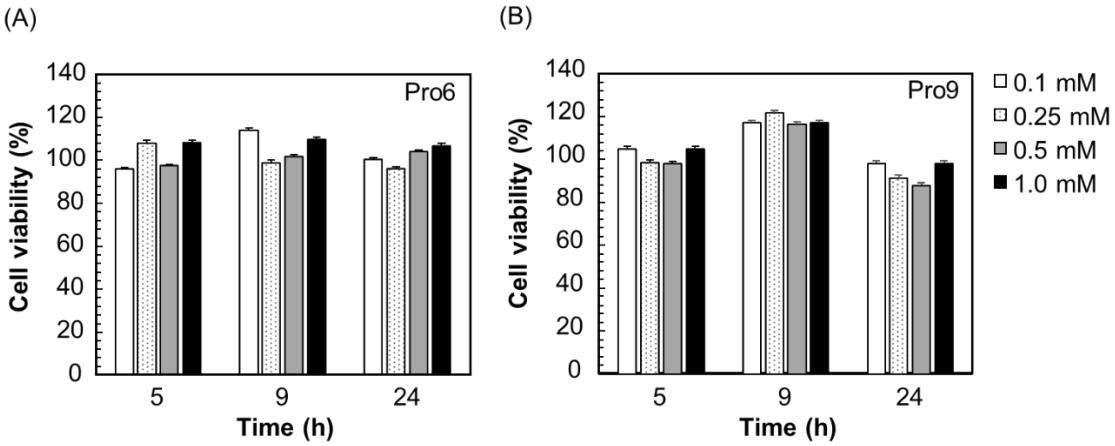


Fig. S5 Viability of fibroblasts (NIH/3T3 clone 5611 transfected with pCAG-LifeAct-TagRFP) after the cultivation of 5, 9, and 24 hours in a culture medium including different concentrations of (A) Pro6 and (B) Pro9. (n=3)