Supplementary Material (ESI) for Soft Matter

Electronic supplementary information (ESI)

A Nano-Fibrous Assembly of Collagen / Hyaluronic Acid for Controlling Cell-Adhesive Properties

Toshinori Fujie[1], *Sho Furutate*[1], *Daisuke Niwa*[1] *and Shinji Takeoka*[1,2*]

[1]Department of Life Science and Medical Bioscience, Graduate School of Advanced Science and Engineering, Waseda University, Tokyo 162-8480, Japan.

[2] High-Tech Research Center, Waseda University, Tokyo 162-8480, Japan.

* Corresponding author at: Department of Life Science and Medical Bioscience, Graduate School of Advanced Science and Engineering, Waseda University, 2-2 Wakamatsu-cho, Shinjuku-ku, Tokyo 162-8480, Japan. Tel.: +813-5369-7324, Fax : +813-5369-7324

E-mail address: takeoka@waseda.jp (S. Takeoka)

Materials

Collagen (COL: M_w = 300,000) was purchased from Koken, Inc. (Tokyo, Japan). Hyaluronan sodium salt (HA: M_w = 1,400) was kindly donated from Shiseido, Inc. (Tokyo, Japan). Polyvinyl alcohol (PVA: M_w = 22,000) was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Fluorescein-isothiocyanate (FITC: Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 5-aminofluorescin (Sigma-Aldrich Co., St. Louis, MO) were used for the fluorescent labeling of COL and HA, respectively. Silicon wafers purchased from KST World Co. (Fukui, Japan), cut to a size of 40 mm x 40 mm, were immersed in a mixture of sulfuric acid/hydrogen peroxide (3/1) for 10 min and then thoroughly rinsed with deionized (D.I.) water (resistivity 18 MΩ cm). COL (0.5 mg/mL) and HA (0.5 mg/mL) solutions were prepared with the acetate buffer (pH 4.7).

Preparation of the transferrable ECM-nanosheet

All routines for ECM-nanosheet fabrication were conducted in a cleanroom (class 10000 conditions) to avoid contamination. The freestanding ECM-nanosheet was fabricated by a dipping LbL method using a modification of the Hilborn's protocol[S1]. The SiO_2 substrate was immersed in a Petri-dish filled with a 10 mL COL solution (0.5 mg/mL, pH 4.7) for 3 min, and then the surface of the substrate was thoroughly rinsed and immersed in an acetate buffer (pH 4.7) for 1.5 min. Next, the substrate was immersed in a HA solution (0.5 mg/mL, pH 4.7) for 3 min, and rinsed with the acetate buffer (pH 4.7) in the same manner as for the COL step. Acetate buffer was chosen to enhance the protonation of COL and deprotonation of HA[S1]. According to the above conditions, the freestanding ECM-nanosheets were prepared by the following procedure: a) repetition of COL and HA multi-layering using the dipping LbL method (3 min for each polyelectrolyte) followed by a rinse in acetate buffer (pH 4.7) after each round of layering; b) termination of LbL in the COL stage followed by drying of the

surface using a flow of nitrogen gas; 3) casting a supporting layer of a 10 wt% PVA solution on the multilayered substrate until the PVA film was completely dried over a period of 12 hrs; 4) bilayered film composed of the ECM-nanosheet and PVA film was peeled from the SiO_2 substrate with tweezers. Finally, immersion of the bilayered film in phosphate buffered saline solution (PBS, pH 7.4) generated a freestanding ECM-nanosheet upon dissolution of the supporting PVA film. Details of this procedure are given in a previous paper[S2].

Collagen Fibrosis in the ECM-nanosheet

Each COL derived microfibril in the ECM-nanosheet was self-assembled into the COL fibril structure by incubation in PBS at 37^{o}C, where the percentage dissociation of 5-amino fluorescein labeled HA[S3] was monitored using a spectrophotometer equipped with a fluorescent microscope (Biorevo BZ-9000, Keyence Co., Tokyo, Japan). The remaining percentage of HA molecules after incubation under physiological conditions was calculated from the fluorescent intensity of the ECM-nanosheet divided by that of the intact glass substrate at each time-interval until 24 hrs (i.e., 100% being the fluorescent intensity in PBS at room temperature before heating at 37^{o}C). The macroscopic surface morphology of the ECM-nanosheet was recorded using a digital camera OLYMPUS C-5050 ZOOM (Olympus Co., Tokyo, Japan). The microscopic surface morphology of the ECM-nanosheet was scanned using an AFM in tapping mode (NanoScale Hybrid Microscope, Keyence Co., Tokyo, Japan). SEM (HITACHI S-4300, Hitachi Co., Tokyo, Japan) images were obtained with a platinum layer generated using an ion-sputtering coater (HITACHI E-1045; 18 mA, 40 s., Hitachi Co.). Thickness of the ECM-nanosheet was measured using a surface profiler (α-step, KLA-Tencor Corp., San Jose, CA). In order to ascertain the existence of the COL fibril, we distinguished the ECM-nanosheet as an F (fibril) and NF (non-fibril)-nanosheet after incubation for 12 hrs.

Mechanical characterization

The bulge test was used for evaluation of the mechanical strength of the ECM-nanosheet. A freestanding ECM-nanosheet floating in water was carefully scooped onto a steel plate containing a 6 mm diameter circular hole in the middle. The plate covered with the ECM-nanosheet was then fixed onto a custom-made steel chamber. The pressure applied to the ECM-nanosheet through the circular hole of the plate was monitored using a digital pressure gauge (Keyence Co., Tokyo, Japan), and the deflection of the ECM-nanosheet was viewed from the side by a stereomicroscope (Olympus Co., Tokyo, Japan) until distortion was apparent. Each measurement for the ECM-nanosheet was performed at least three times. In order to determine the ultimate tensile strength, the ultimate tensile elongation and the elastic modulus of the ECM-nanosheets, the following equations were used:

$$\sigma = (P \times a^2)/(4 \times h \times d) \quad (1)$$

$$\varepsilon = (2 \times d^2)/(3 \times a^2),\ E = \sigma/\varepsilon \quad (2)$$

where σ, P, a, h, d, ε and E represent tensile stress, applied pressure, radius of the circular hole of the steel plate, thickness, deflection, tensile strain and elastic modulus about the ECM-nanosheet, respectively.

Cell culture on the ECM-nanosheet

NIH-3T3 (mouse fibroblasts cell line) cells were cultured in 10% fetal bovine serum including penicillin streptomycin solution (1%) (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Cell growth was performed in an atmosphere of 5% CO_2 at 37^{o}C. After reaching confluence, the cells were suspended with a 0.05 wt% trypsin–0.53 mmol/L EDTA-4Na solution with phenol red (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The FITC-labeled ECM-nanosheet supported by the PVA film was cut into the desired shape with scissors and then transferred onto either a 60-mm diameter polystyrene Petri-dish (BD Falcon,

Co., Franklin Lakes, NJ), glass-bottomed dish (Asahi Glass Co. Ltd., Chiba, Japan) or a stainless steel mesh with a 0.6 mm x 0.6 mm lattice (Tokyu Hands, Co. Ltd., Tokyo, Japan). The PVA film was dissolved in distilled water and gently dried with a flow of nitrogen gas onto a surface used for cell culture (1 x 10^5 cells/cm^2). In order to analyze the adhesive properties of the surface, cells on the ECM-nanosheet were fixed with a 4% paraformaldehyde solution and stained with Alexa Fluor® 594 phalloidin (Invitrogen Co., Carlsbad, CA) and DAPI (Wako Pure Chemical Industries, Ltd., Osaka, Japan) after 4 hrs or 24 hrs of culture .

Statistical analyses

The data are presented as mean values ± SD. Statistical analyses were performed using a Stat View 4.02J software package (Abacus Concepts, Berkeley, CA). The cell elongation ratio was compared using the ANOVA test with $*p < 0.05$ and $**p < 0.01$ set as the level of statistical significance.

Reference

S1. J. A. Johansson, T. Halthur, M. Herranen, L. Söderberg, U. Elofsson, J. Hilborn, *Biomacromolecules*, 2005, **6**, 1353.

S2. T. Fujie, J-Y. Park, A. Murata, N. C. Estillore, M. C. R. Tria, S. Takeoka, R. C. Advincula, *ACS Appl. Mater. Interfaces*, 2009, **1**, 1404.

S3. M. Nakagawa, M. Tanaka, T. Miyata, *Ophthal. Res.* 1997, **29**, 409.

Supplementary Figures (S1-S5)

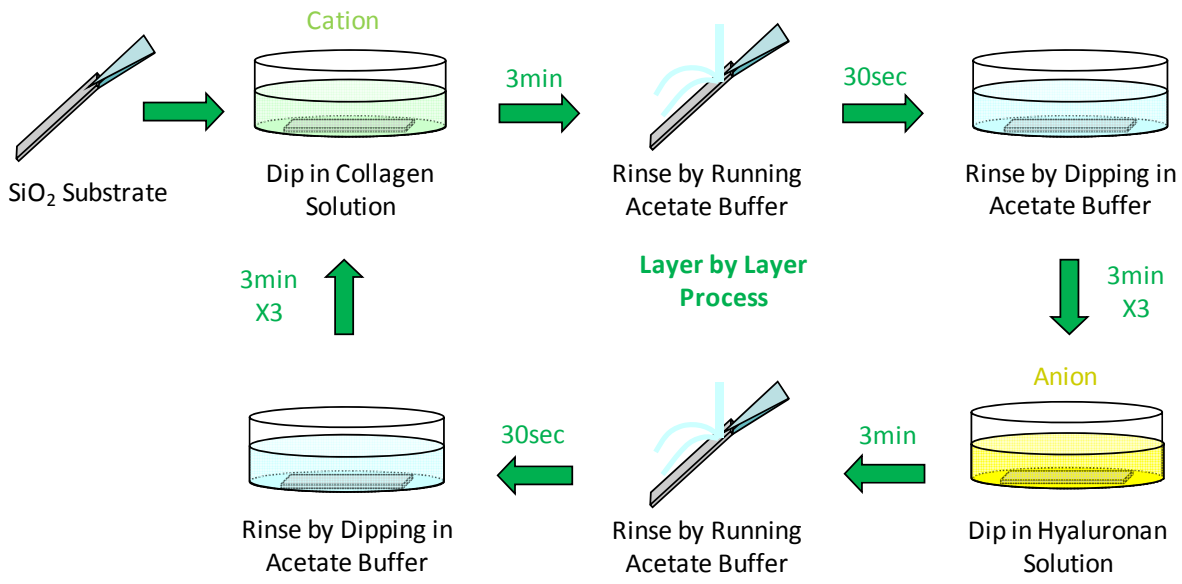

Fig. S1 Preparative scheme for COL and HA assembled LbL film.

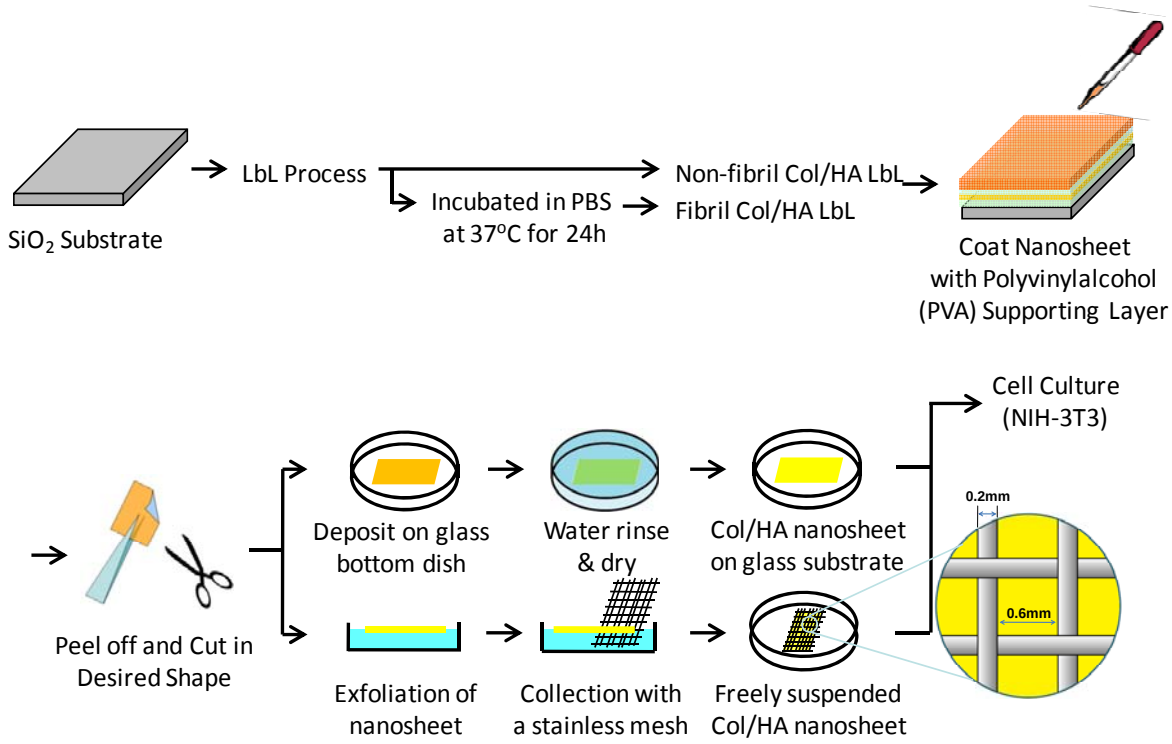

Fig. S2 Preparative scheme for the ECM-nanosheet including the transfer method.

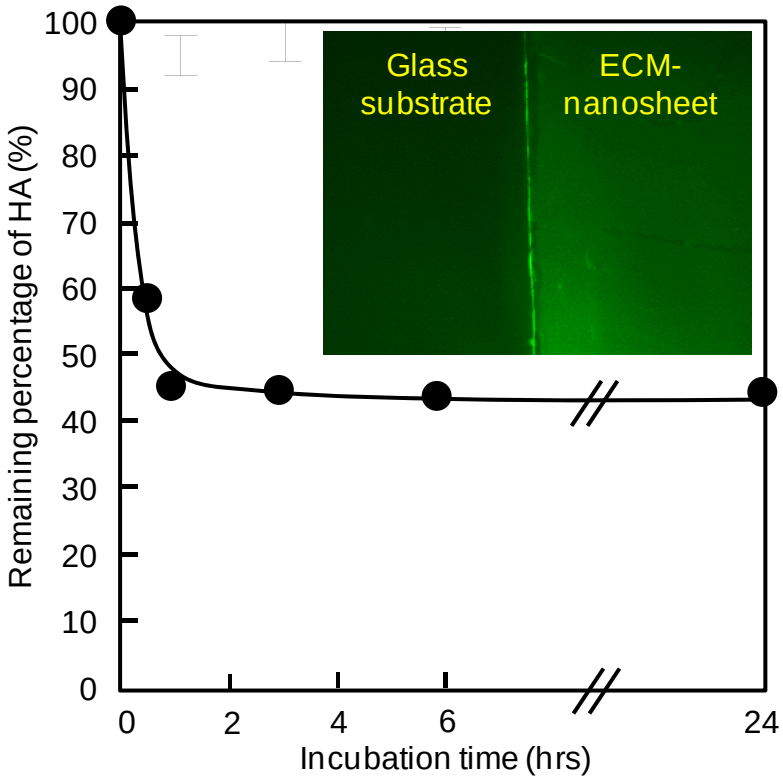

Fig. S3 Remaining percentage of HA molecules in the ECM-nanosheet after incubation under physiological conditions (pH 7.4, 37^{o}C) with respect to the fluorescent intensity of a 5-amino fluorescein labeled HA. Inset shows a typical edge image of the ECM-nanosheet containing the fluorescent HA.

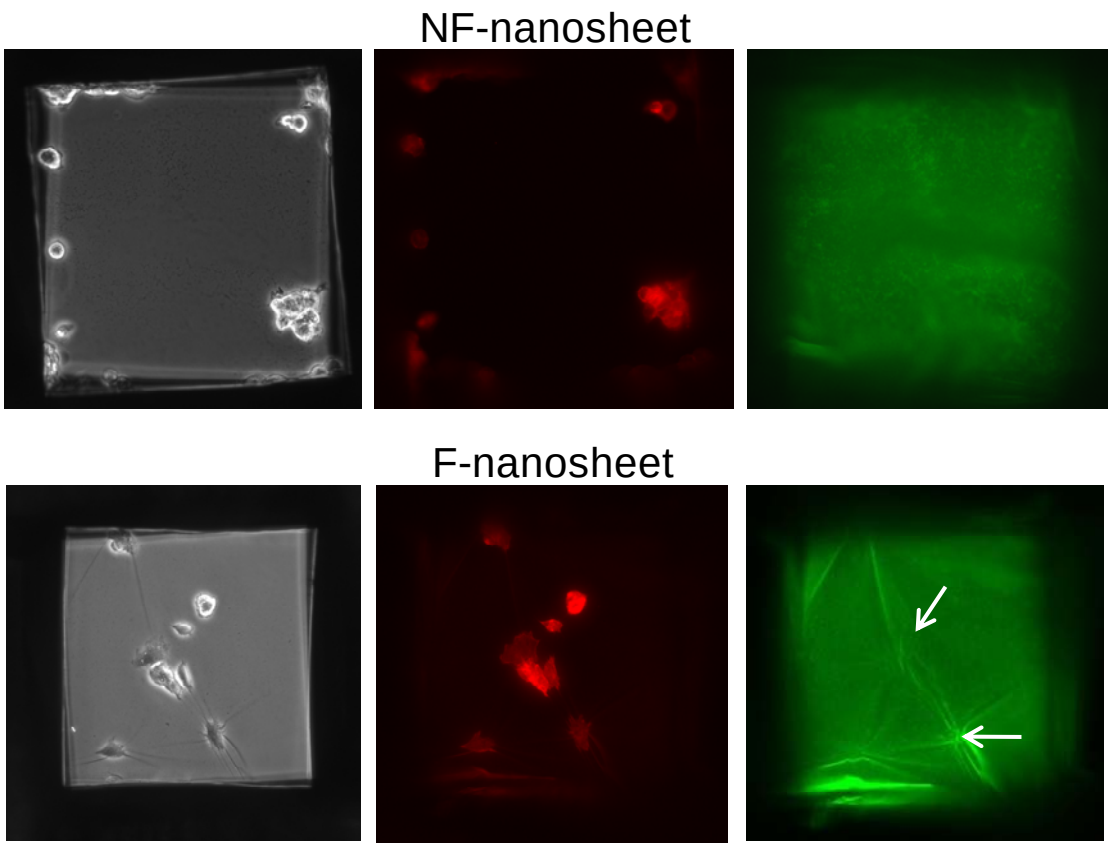

Fig. S4 Separated microscopic image of the ECM-nanosheet interacting with the NIH-3T3 at 4 hours; optical images (left), fluorescent images of actin stained by Alexa Fluor® 594 phalloidin (middle) and ECM-nanosheet stained with FITC (left). On the bottom-right panel, corrugations of the ECM-nanosheet caused by cellular contraction are observed (highlighted by the arrows).

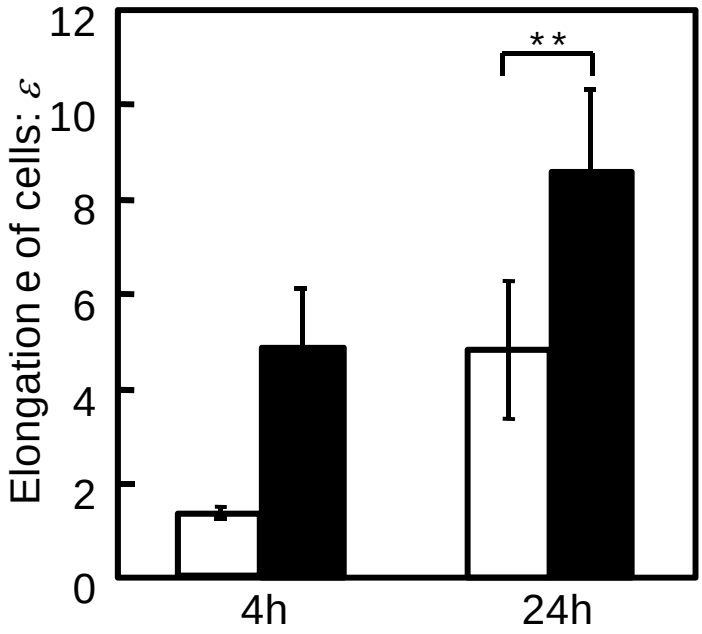

Fig. S5 Time-course analysis of the cell elongation ratio for NIH-3T3, adhered on the F (solid bar) or NF-nanosheet (blank bar) supported by the stainless mesh. ** $p<0.01$.