

**Using Surfaces to Modulate the Morphology and Structure of Attached Cells – a Case of Cancer
Cells on Chitosan Membranes**

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Experimental details

Preparation of Pristine Chitosan Membranes with Various Molecular Weights. Low- (50-190 kDa), medium- (190-310 kDa), and high- (310-375 kDa) molecular-weight chitosan powders purchased from Sigma–Aldrich (St. Louis, MO, USA) with a deacetylation degree of ~85% were used in this study. For each molecular weight chitosan, 1% (w/v) chitosan solution was prepared by dissolving chitosan powders in 1% (v/v) acetic acid. The solution was continuously stirred with a sterile magnetic bar for 6 hours and undissolved impurities and foams were removed via vacuum filtration and a Whatman No. 541 filter paper (0.22 µm pore size). The chitosan solution was poured into a petri dish and dried in an oven at 60°C for ~6 hours to evaporate the solvent. Next, the chitosan membrane was immersed in 0.1 M sodium hydroxyl solution for 12 hours. The pristine chitosan membranes of various stiffness were finally rinsed with deionized water until neutral pH value was achieved.

Preparation of Chitosan Membranes with Different Functional Groups. The chitosan membrane was hydroxylated by O₂ plasma via molecular vapor deposition system (MVD 100, Applied Microstructures Inc., USA) at 30 W under 20 Pa for 15 minutes (to obtain an O₂-plasma-treated chitosan membrane). After the formation of hydroxyl groups, the FDTS-grafted chitosan membrane was made by molecular self-assembly of FDTS with fluorine groups.^[1]

Preparation of Chitosan Membranes with Nanotextured Surfaces. The silicon nanosponges were made on monocrystalline silicon wafers (100) using chemical etching.^[1] The wafer was dipped into a mixture of hydrogen fluoride (HF, 49% wt.), hydrogen peroxide (H₂O₂, 30% wt.), and silver nitrate aqueous solution (AgNO₃, 0.01 M) for 3 minutes at room temperature. Silver ions (Ag⁺) in this mixture reduced on the monocrystalline silicon surface because the electronegativity of silver was higher than silicon. The formed silicon dioxide during this process was further etched by HF (49% wt.), in order to obtain the porous structures on the monocrystalline silicon surface. The silver film wrapped on the

silicon nanosponges was removed by diluted H₂O₂; silicon nanosponges were then rinsed with deionized water. The chitosan membrane with a nanotextured surface (nanosponges) was fabricated by casting chitosan solution onto silicon nanosponges (i.e., silicon nanosponges as the mold) and then drying the chitosan solution on silicon nanosponges in the oven. The chitosan membrane with nanosponges was dipped into 0.1 M sodium hydroxyl solution for 8 hours, carefully peeled from silicon nanosponges, and then rinsed with deionized water.

Scanning Electron Microscopy (SEM). The morphology of various chitosan membranes was examined with high-resolution field-emission SEM – Ultra 55 (Carl Zeiss SMT AG, German). The chitosan membranes were dried in air and then sputtered with gold at a current of 15 mA for 3 minutes by using an ion sputter.

Surface Hydrophobicity. The surface hydrophobicity of chemically modified and nanotextured chitosan membranes was evaluated by the contact angle measurement via a contact angle meter – MD 100SB (Sindatek Instruments Co., Taiwan). A droplet of deionized water (volume = 2 µL) was placed by a motor-driven syringe at room temperature on a chitosan membrane, and then the static contact angle was determined to derive the hydrophobicity of this chitosan membrane after the droplet on a chitosan membrane was stabilized. The result was obtained through the average of five independent measurements.

Atomic Force Microscopy (AFM). The atomic force microscope employed in this study was NanoWizard BioScience AFM (JPK Instruments, Germany), for investigating the topography of pristine and nanotextured chitosan membranes (under the contact mode). We employed rectangular shaped silicon nitride based PNP-DB cantilevers (Nanoworld AG, Switzerland) rated for a nominal spring coefficient 0.06 N/m and resonance frequency 17 kHz. The cantilevers were cleaned with

ethanol and immediately rinsed with de-ionized water before use. In addition, the stiffness of chitosan membranes made via chitosan powders with different molecular weights was also evaluated through the force curve measurement under the contact mode.

X-ray Photoelectron Spectroscopy (XPS). In order to analyze the molecular bonding condition on pristine, O₂-plasma-treated and FDTS-grafted chitosan membranes, a high-resolution X-ray photoelectron spectrometer was used to examine the surfaces of these chitosan membranes with various surface modifications. HRXPS PHI Quantera (ULVAX-PHI, Japan) was equipped with the light source of scanning monochromated Al anode and the energy analyzer of 180° spherical capacitor analyzer with a 32-channels detector. XPS analysis was performed at room temperature, with 1486.6 eV X-ray from the Al K α line and a 45° incident angle measured from the sample surface. Samples were loaded into an ultrahigh vacuum chamber, approximately 5×10^{-10} torr, of an M-probe surface spectrometer.

Cell Culture. Mouse fibroblasts (NIH-3T3), and their transgenic derivatives NIH-3T3 cells containing an inducible *Ha-ras*^{val12} oncogene regulated by an Escherichia coli lac repressor were used in this study.^[2] Both cells were maintained in 90% Dulbecco's Modified Eagle's Medium supplemented with 10% calf serum, 4 mM L-glutamine, 100 U/mL penicillin and 1.5 g/L sodium bicarbonate at 37°C in a humidified 5% CO₂ incubator. Isopropyl-1-thio- β -D-galactopyranoside (IPTG), a non-metabolizable lactose analogue, with the concentration of 5 mM, was used to induce the expression of mutated *Ha-ras* transgene in NIH-3T3 fibroblasts. Cells were trypsinized with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) and re-seeded onto the sterilized chitosan membranes at a density of 5×10^4 cells/mL for 12, 24, and 48 hours to perform the experiments and examinations as described, respectively. Human cervical carcinoma cells (HeLa) were used and maintained in 90% Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100 U/mL penicillin and 1.5 g/L sodium bicarbonate at 37°C in a humidified 5% CO₂ incubator to

study single cell patterning with the chemical modulator examinations.

Staining and Imaging Analysis with Fluorescence Microscopy. NIH-3T3 fibroblasts and their *Hras*-transformed progeny were cultured on various chitosan membranes for a period of time (12, 24, and 48 hours in this study), followed by three phosphate buffered saline washes. Cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature, followed by three phosphate buffered saline washes. The chitosan membranes were then further dipped in 0.1% Triton X-100 for 10 minutes to permeabilize the cell membranes. After three phosphate buffered saline washes, rhodamine-conjugated phalloidin (Invitrogen, Carlsbad, CA, USA) was used to label actin filaments, while 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA, USA) was used to stain DNA in nuclei, respectively. For the cytoskeletal organization analysis, Deoxyribonuclease I (Invitrogen, Carlsbad, CA, USA) was used to label G-actin, while Alexa Fluor 488 phalloidin (Invitrogen, Carlsbad, CA, USA) was used to stain F-actin. After blocking with 1% BSA, mouse anti-integrin $\alpha 1\beta 1$ primary antibody (Abcam, Cambridge, UK) and goat polyclonal secondary antibody to mouse IgG - H&L (Chromeo 488) (Abcam, Cambridge, UK) were used to stain integrin $\alpha 1\beta 1$ in NIH-3T3 fibroblasts. In addition, rhodamine fibronectin placed over the chitosan-based nanosponges (10 μg in 1 mL PBS; Cytoskelton Inc., Denver, CO, USA; No.: FNR01) for at least one hour (through physical adsorption) was used to provide specific chemically-based ligands between integrins in NIH-3T3 fibroblasts and chitosan-based nanosponges (with fluorescently-labeled extracellular matrix molecules). For single cell patterning, HeLa cells were cultured on single cell chitosan array for 12 hours, followed by three phosphate buffered saline washes. Cells were fixed, permeabilized and stained with Deoxyribonuclease I and Alexa Fluor 488 phalloidin for G-actin and F-actin, respectively. Apoptosis assay was conducted by staining Alexa Fluor 488-conjugated Annexin V (Invitrogen, Carlsbad, CA, USA) to label apoptotic cells after treating with a chemical modulator, cytochalasin D (10 μM , Sigma–Aldrich, St. Louis, MO, USA), for 1 hour. Then the cells were fixed to substrates with 4% paraformaldehyde for 15 minutes and

further permeabilized with 0.2% Tween-20 for 1 minute. Finally, the substrates for single cell patterning and apoptosis assay were rinsed and mounted with mounting medium containing with DAPI. The cells were then examined under an epi-fluorescence microscopy (Zeiss Axio Observer, German) equipped with a 63X (NA = 1.4) oil immersion objective and a 10X eyepiece. For probing the interactions between integrins (in NIH-3T3 fibroblasts) and single chitosan-based nanosponges (treated with rhodamine fibronectin), the fibroblasts cultured on these chitosan-based nanosponges were examined with laser confocal scanning microscopy (Zeiss LSM 700, German) equipped with a 100X (NA = 1.4) oil immersion objective and a 10X eyepiece through an appropriate integrin immunostaining (that we developed in this study).

Statistical Analysis. Image analysis of projected cell area and cell density (cell proliferation) on the chitosan membranes were performed with ImageJ (public software from National Institutes of Health; <http://rsbweb.nih.gov/ij/>). The fluorescence intensity of expressed integrin $\alpha1\beta1$, F-actin and G-actin in individual cells were also quantified using ImageJ. Results in this study were reported as the mean \pm standard deviation and were analyzed using Student's t-test for the statistical difference.

References

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