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Formation of nanofilms on cell surfaces to improve the insertion efficiency of a nanoneedle into cells

Yosuke Amemiya [a], Keiko Kawano [b], Michiya Matsusaki [c], Mitsuru Akashi [c], Noriyuki Nakamura [b], Chikashi Nakamura [a,b,*]

[a] Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Central 4, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8562, Japan
[b] Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology, 2-24-26 Naka-cho, Koganei, Tokyo 184-8588, Japan
[c] Department of Applied Chemistry, Graduate School of Engineering Science, Osaka University, 2-1 Yamada-oka, Suita, Osaka 565-0871, Japan

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ABSTRACT

A nanoneedle, an atomic force microscope (AFM) tip etched to 200 nm in diameter and 10 μm in length, can be inserted into cells with the aid of an AFM and has been used to introduce functional molecules into cells and to analyze intracellular information with minimal cell damage. However, some cell lines have shown low insertion efficiency of the nanoneedle. Improvement in the insertion efficiency of a nanoneedle into such cells is a significant issue for nanoneedle-based cell manipulation and analysis. Here, we have formed nanofilms composed of extracellular matrix molecules on cell surfaces and found that the formation of the nanofilms improved insertion efficiency of a nanoneedle into fibroblast and neural cells. The nanofilms were shown to improve insertion efficiency even in cells in which the formation of actin stress fibers was inhibited by the ROCK inhibitor Y27632, suggesting that the nanofilms with the mesh structure directly contributed to the improved insertion efficiency of a nanoneedle.

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1. Introduction

Analyzing intracellular information *in vivo* is a prominent way of understanding the cellular function and the techniques employed are significant for development of novel biomedical applications. The recent progress of nanotechnology has promoted the development of the needle-shaped devices for molecular delivery into cells and analysis of intracellular information with minimum cell damage. Carbon or boron nitride nanotube tips were used for the introduction of quantum dots into a single cell and motion analysis of the quantum dots in the intracellular environments was performed [1,2]. The use of arrays of the vertically aligned carbon or silicon nanowires, carbon nanofibers and carbon nanosyringes enabled the simultaneous introduction of functional molecules into multiple cells [3–6]. Electrophysiological analysis in the intracellular environment can be performed with needle-shaped nanoelectrodes partially coated with insulating materials [7–9]. Recently, the endoscopic approaches using the needle-shaped devices have been proposed. A multiwalled carbon nanotube tip coated with gold particles was used as a probe in surface-enhanced Raman spectroscopy [10] and an SnO_2 nanowire was used as an optical waveguide to illuminate the excitation light at a high spatial resolution in the cells [11].

We have been developing techniques for cell manipulation and analysis using an atomic force microscope (AFM) with a silicon "nanoneedle", which is a silicon AFM tip etched to a cylindrical shape with a 200 nm diameter and a 10 μm length using a focused ion beam. The nanoneedle can be efficiently inserted into cells [12,13] and the use of a functionalized nanoneedle allows highly efficient DNA transfection [14] and detection of intracellular mRNA [15] and cytoskeletal proteins [16]. The use of AFM for inserting nanoneedle into cells has the advantage that the insertion events can be confirmed by analyzing the force response during the insertion process [13]. Techniques for the validation of the insertion events are essential for surveying intracellular information without artifacts derived from the false insertion. It is reported that the nanosized electrodes with a 150 nm diameter and a 1.5 μm length cannot penetrate the cell membrane of HL-1 cells (a mouse cardiac muscle cell line), which had been seeded on a nanosized electrode array, without applying external voltage [9]. Similarly, we found that some cell lines showed low insertion efficiency of the nanoneedle using our manipulation apparatus. We reported that the insertion efficiency of the nanoneedle varies depending on the cell species and that stress fibers and actin meshwork comprising the plasmalemmal undercoat is essential for the nano-

* Corresponding author at: Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Central 4, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8562, Japan. Fax: +81 29 861 3048.
E-mail address: chikashi-nakamura@aist.go.jp (C. Nakamura).

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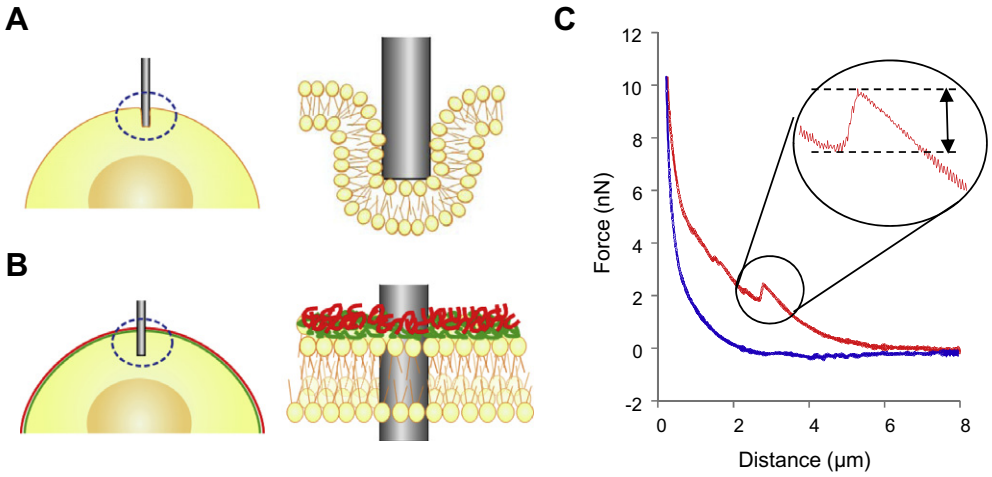

Fig. 1. Schematic illustrations of the nanoneedle insertion into cells, without (A) and with (B) the FN-G or FN-DS nanofilms on cell surfaces. Green lines indicate fibronectin and red lines indicate gelatin or dextran sulfate. The force drop event shown in the force curve was obtained as a nanoneedle was extended to and then retracted from cells (C).

needle insertion [17]. This finding led us to conceive the idea that mesh structures formed on a cell surface would improve the insertion efficiency of the nanoneedle (Fig. 1A and B). The nano-sized mesh-like morphology of the thin films, which consist of extracellular matrix molecules, can be form on the cell surface using a layer-by-layer technique [18,19]. The cytotoxicity of the nanofilms is low and therefore the nanofilms are good candidates as nanostructures formed on the cell surface for the improvement in the insertion efficiency of the nanoneedle. Here, we investigated the effect of the nanofilms formed on cell surfaces on the insertion efficiency of the nanoneedle.

2. Methods

2.1. Cell culture

1×10^5 cells of mouse embryonic fibroblast cell line, Balb3T3 (RIKEN Cell Bank, Tsukuba, Japan) were seeded on collagen-coated 35 mm glass-bottom culture dishes (φ 27 mm, Asahi Glass, Tokyo, Japan) and cultivated in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), overnight. The collagen-coated glass-bottom culture dishes were prepared by putting the 0.05 mg/mL collagen (Collagen Type I from calf skin, Sigma–Aldrich, St. Louis, MO, USA) solution on the glass surface and then incubating for 1 h at room temperature, followed by washing with PBS. 1×10^5 cells of mouse P19 embryonic carcinoma cells (Dainippon Sumitomo Pharma, Osaka, Japan) were cultivated in polystyrene Petri dishes (φ 90 mm, As one, Tokyo, Japan) with α-MEM (Invitrogen) containing 10% FBS, 2 mM GlutaMAX (Gibco), Gentamicin–Amphotericin B (10 and 0.25 μg/mL, Cascade Biologics, Portland, OR, USA) for 4 days in the presence of 1 μM all-trans retinoic acid (RA) (Sigma–Aldrich). The resulting cell aggregates were treated with 0.025% trypsin and 0.01% EDTA solution for 10 min at room temperature and then centrifuged to form a pellet. The cell pellet was dispersed in the culture media and the cells were seeded onto the plastic slips (Cell Desk, Sumitomo Bakelite, Tokyo, Japan) coated with polyethyleneimine (Sigma–Aldrich). The polyethyleneimine-coated plastic slips were prepared by incubating coverslips in 0.02% polyethyleneimine solution for 1 h, followed by washing with PBS. The cells were cultivated on the plastic slip for 2–6 days. All cells were cultured at 37 °C under 5% CO_2.

2.2. Formation of nanofilms on cell surfaces

The nanofilms composed of fibronectin (fibronectin from Bovine Plasma, Wako, Osaka, Japan) and gelatin (Wako) (FN-G nanofilm) and fibronectin and dextran sulfate (Wako) (FN-DS nanofilm) were formed on cell surfaces using a layer by layer technique, as described previously [18]. Briefly, the cultured Balb3T3 or differentiation-induced P19 cells were incubated in PBS containing 0.2 mg/mL fibronectin for 1 min at room temperature and then washed with PBS. The cells were incubated in PBS containing 0.2 mg/mL gelatin or sodium dextran sulfate for 1 min at room temperature and then washed with PBS. The consecutive treatments with fibronectin and gelatin were conducted 1, 3, 5 and 10 times to form FN-G nanofilms (FN-G1, FN-G3, FN-G5, and FN-G10) on cell surfaces. The consecutive treatments with fibronectin and dextran sulfate were conducted once to form FN-DS nanofilms (FN-DS1). The cells coated with the nanofilms were cultivated for 24 h and used for the following experiments.

2.3. Treatment of cells with Y27632

The Balb3T3 cells coated with FN-G3 were incubated in the culture media containing 100 μM Y27632 (Calbiochem, Darmstadt, Germany) for 1 h at 37 °C, followed by washing with PBS.

2.4. Immunostaining

P19 cells treated with RA were fixed with 4% formaldehyde (Wako) for 15 min, washed with PBS and treated with 0.1% Triton X-100 (Nacalai Tesque, Kyoto, Japan) in PBS for 3 min. The cells were then washed with PBS 3 times and treated with 4% BLOCK ACE (Dainippon Sumitomo Pharma, Osaka, Japan) for 1 h at room temperature. The cells were incubated with PBS containing 15 nM anti-Neurofilament-L antibody (Neurofilament-L (C28E10) Rabbit mAb, Cell Signaling Technology, Danvers, MA, USA) for 1 h at room temperature and then washed with PBS. The cells were incubated with PBS containing 15 nM Alexa Fluor 568-labeled goat anti-rabbit IgG (Invitrogen) for 1 h at room temperature and then washed with PBS. Consecutively, the cells were incubated with PBS containing 15 nM anti-βIII tubulin antibody (Neuronal Class III β-Tubulin (TUJ1) Mouse Monoclonal Antibody, Covance, Berkeley, CA, USA) for 1 h at room temperature. The cells were washed with PBS and then incubated with PBS containing 15 nM Alexa

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