Short communication

A molecular delivery system by using AFM and nanoneedle

Sung Woong Han\textsuperscript{a,b}, Chikashi Nakamura\textsuperscript{a,b,*}, Ikuo Obataya\textsuperscript{a}, Noriyuki Nakamura\textsuperscript{a,b}, Jun Miyake\textsuperscript{a,b}

\textsuperscript{a} Research Institute for Cell Engineering (RICE), National Institute of Advanced Industrial Science and Technology (AIST), 3-11-46 Nakaji, Anagukicho, Hongo 166-0074, Japan
\textsuperscript{b} Division of Biotechnology and Life Science, Tokyo University of Agriculture and Technology, 2-24-26 Naka-cho, Koganei, Tokyo 184-8588, Japan

Received 20 May 2004; received in revised form 4 August 2004; accepted 11 August 2004

Available online 22 September 2004

Abstract

We developed a new low invasive cell manipulation and gene or molecule transfer system in a single living cell by using an atomic force microscope (AFM) and ultra thin needle; a nanoneedle. DNA was immobilized on the surface of the nanoneedle by covalent bonding and avidin–biotin affinity binding. Immobilization of DNA on the nanoneedle was confirmed by measuring the unbinding force between avidin and biotin. The DNA-immobilized nanoneedle was successfully inserted into HEK293 cells. Though TO-PRO-3 iodide staining experiments using confocal microscopy, we observed the immobilized DNA on the surface of the nanoneedle, which was retained after 10 times insertions to and evacuations from a living cell.

© 2004 Elsevier B.V. All rights reserved.

Keywords: Cell manipulation; Gene transfer; AFM; Force–distance curve; Avidin–biotin unbinding force

1. Introduction

Molecular delivery technologies to target cells are fundamental requirements in biotechnology and biochemistry. Many kinds of molecular or gene delivery techniques to target cells have been developed. Recent examples include viral vector-mediated systems (Garcia-Ocana et al., 2003; Kasparov et al., 2004), polymer-mediated systems (Suzuki et al., 2004; Zelkin et al., 2003), liposome complexes (Hattori et al., 2004; Hwang et al., 2001; Torchilin et al., 2003), chitosan-mediated nanoparticles (Corsi et al., 2003; Mao et al., 2001), electroporation (DeBruin and Krassowska, 1999), and microinjection (Ikuta et al., 2004; Tsuha et al., 2003). It is very important point that the most proper method must be selected in each research purpose because of their merits and weaknesses. Although microinjection is the only single cell manipulation system and the simplest way to transfer DNA, it has also the disadvantage that the treated cells suffer severe damage of the penetration by the large-scaled capillary (∼1.0 μm) after manipulation (Bird et al., 2003). Owing to the damage, the survival rate (Ito et al., 1998) and efficiency of microinjection is very low and the applications of microinjection are generally limited.

Since the invention of the AFM (Binning et al., 1986), amazing progress has been made in the imaging and manipulation of bio-molecules. AFM topographs of plant (McMaster et al., 1996; Winfield et al., 1995), insect (Jondle et al., 1995; Vesenka et al., 1995), and human chromosomes (DeGrooth and Putman, 1992; Rasch et al., 1993) with nanometer lateral resolution and subnanometer vertical resolution have been acquired by various groups.

A lot of experimental effort has also gone into studying receptor/ligand interactions, for example the small ligand biotin interacting with the closely related proteins streptavidin or avidin (Florin et al., 1995; Merkel et al., 1999; Moy et al., 1994), protein/protein interactions (Vinckier et al., 1998), and
interactions between complementary strands of DNA (Rief et al., 1999), etc.

We propose a new application for AFM involving a cell manipulation system. The objective molecules, for example, genes or proteins, are immobilized on an ultra thin needle. The gene-immobilized needle is inserted into a single living cell. The cell damage by insertion can be reduced compared to microinjection because the diameter of needle is quite thin, 200 nm, a so-called nanoneedle. The nanoneedle is one-tenth diameter of a capillary used in microinjection. The accurate three-dimensional control of the needle is possible by using an AFM system. The information from the insertion of the needle into the cell can be monitored as the signal of the force applied to the cantilever. Moreover, the inserted depth of the nanoneedle can also be monitored and controlled by using the AFM force–distance curve. This is a very attractive point in cell science because it makes possible the accurate delivery of nucleic acids and proteins into the nucleus or cytoplasm.

In this work, we fabricated a nanoneedle of 200 nm in diameter and DNA was immobilized on the surface of nanoneedle using covalent bonding and avidin–biotin affinity binding. Immobilization of DNA on the nanoneedle was confirmed by measurement of the unbinding force between avidin and biotin, and through observation of the needle surface using confocal laser scanning microscopy after the insertion.

2. Materials and methods

2.1. Cell culture

HEK293 cells were grown in DMEM supplemented with 10% FBS and PSA. The cells were maintained in a humidified incubator under 5% CO2 at 37 ◦C. Flasks containing cells were treated with trypsin and then centrifuged to form a pellet. The cell pellet was dispersed and the cells were plated on a collagen coated 35 mm tissue culture dish (IWAKI, Tokyo, Japan) or 50 mm glass-bottom culture dishes (MatTek, Ashland, USA). The glass-bottom culture dishes was coated with 50 µg/mL of collagen in 0.02N acetic acid by incubating for 1 h, following careful washing with phosphate buffered saline.

2.2. Chemicals and DNA

3-Mercaptopropyltrimethoxysilane (MPTMS) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). N-[6-Maleimidocaproyloxy)succinimide (EMCS) was purchased from Dojindo Laboratories (Kumamoto, Japan). TO-PRO-3 iodide (642 nm/661 nm) was purchased from Molecular Probes, Inc. (Eugene, OR, U.S.A.).

The plasmid pQBI25 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing the promoter and the GFP structural gene was amplified by PCR using biotinylated primers.

2.3. Atomic force microscopy (AFM)

A molecular force probe (MFP-1D, Asylum Research, Santa Barbara, CA, U.S.A.) was used for all the force measurements and cell manipulations. In order to observe cells while operating of the MFP, it was placed on the stage of an inverted microscope (IX-70, Olympus, Tokyo, Japan). This combination allows us to observe cells on the culture dish under the head-unit of the MFP. Images can be obtained using a highly sensitive CCD (DP-70, Olympus) equipped on the microscope. Heaters and a CO2 regulator were equipped to maintain the conditions of the cells. The apparatus is covered with a sound-proofing hood to reduce interference from acoustic noise.

2.4. Fabricating of nanoneedle

The nanoneedles were fabricated by focused ion beam (FIB) etching of pyramidal Si AFM tips that have spring constants approximately 0.2 N/m (CONT, Nanosensors, Neuchatel, Switzerland). At first, the FIB was sequentially focused on two areas of pyramid tip so as to leave a thin area on the tip. Then the etched tip was rotated 90◦ to face the resulting triangular plate shape into the ion beam and was etched in the same manner as the first etching. The needle was tilted 10◦ with respect to the center axis of the tip in order to compensate for the mounting angle of the holder.

2.5. Preparation of nanoneedle and mica substrate

The surface of the nanoneedle was treated with 2% MPTMS and 2% MQ water in ethanol for 30 min after cleaning and oxidizing using an ozone cleaner. The nanoneedle was further treated with 1 mM EMCS for 30 min. The succinimidy1 nanoneedle was soaked in avidin solution of 1 µM in HEPES buffer (pH 7.4). Then, the biotinylated GFP DNA fragment solution was dropped on to the cantilever and the nanoneedle to immobilize the biotinylated DNA fragment on the streptavidin-coated probe. Streptavidin was also immobilized following the same method as for the mica substrate.

2.6. Force measurements by AFM

The head-unit of the MFP-1D was mounted on the stage of the microscope, and the cantilever was moved down close to the streptavidin-immobilized mica substrate in HEPES buffer whilst observing the cantilever and the mica by CCD. The measurements were conducted using Si cantilevers with a spring constant of 0.2 N/m. The spring constant of each cantilever was calculated using the thermal method. The force was measured during approaching and retraction with a piezo speed of 1 µm/s.
The force–distance curve for inserting the DNA-immobilized nanoneedle into the cell was measured during approaching and retraction on a cell with a piezo speed of 4 \( \mu m/s \).

### 2.7. Confocal microscopy

For confocal laser scanning microscopy, the DNA-immobilized nanoneedle was stained with 0.1 \( \mu g/ml \) TO-PRO-3 iodide in PBS. Images were taken using a FluoView 300 (Olympus), a confocal laser scanning imaging system equipped an inverted microscope (IX 71, Olympus) using a 60\( \times \) oil immersion objective. Excitation of the TO-PRO-3 iodide labeled nanoneedle was achieved using a He–Ne (633 nm) laser. Two high-sensitivity photomultiplier tubes (PMTs) was set at 650 V. Confocal sections were taken every 0.2 \( \mu m \). Digital image recording was performed using the FluoView software (Olympus).

### 3. Results and discussion

Normal silicon pyramidal tips were sharpened into needle-shape by FIB etching. The diameters of nanoneedles were controlled to 200 nm in diameter and 6 \( \mu m \) in length (Fig. 1). All the needles were fabricated with a cylindrical shaped needle body.

Fig. 2 shows a schematic illustration of the nanoneedle and mica substrate. Immobilization of DNA on the nanoneedle was confirmed by measuring the unbinding force between avidin and biotin. In the case of Fig. 3A, an avidin-immobilized nanoneedle was used. An excess amount of the biotinylated DNA fragment was subjected to immobilization on the needle surface. This meant that there must have been free biotin at the other terminus of the DNA. A second contact of the tip of the needle on the substrate in buffered solution caused binding between biotin and the substrate avidin, and a DNA bridge(s) between the tip of the needle and the substrate was formed. Subsequent retraction of the needle ruptured the DNA bridge. During the retraction process it was obvious that only large forces of over 200 pN applied to the cantilever were observed in the measurements using the DNA-immobilized needle. The averaged maximum force was 270 \( \pm \) 97 pN consistent with previously reported data of the unbinding force of avidin–biotin interaction (Zhang and Moy, 2003). On the other hand, almost no interaction force was measured using a needle without an attached DNA fragment (Fig. 3B). The observed unbinding force suggested that the DNA fragments were immobilized via avidin–biotin interactions and only one terminal end of the linear fragment could be immobilized on
Fig. 4. Typical force–distance curves of nanoneedle insertion to a HEK293 cell. The curve in (A) and (B) were obtained using a DNA-immobilized nanoneedle and a DNA-immobilized unetched AFM tip, respectively.

the needle by controlling the concentration of DNA in the immobilization step.

Fig. 4A shows a typical force–distance curve for the insertion and evacuation process of a DNA-immobilized nanoneedle to and from a HEK293 cell. In the approach, the repulsive force started to increase from the point that the needle head made contact with the cell surface. After about 500 nm of indentation from the contact point, the force relaxed suddenly and hereafter the force did not increase and fluctuated for the next 5 µm of the approach process. After this fluctuating section, a steep force increase was observed. The displacement of the nanoneedle in the fluctuating period is equal to the length of the nanoneedle. These features of the force curve were observed reproducibly. We presume that the force-relaxed point, the force-fluctuating period and the final force increase correspond to the point that the needle head penetrates into a cell membrane, the period that the nanoneedle penetrates through the cell membrane and the point that the base of the needle contacts the cell surface, respectively. In other words, we interpreted the presence of a force-relaxed point and a force-fluctuated period in the force–distance curve as a measure of success of the needle penetration. The second force-relaxed point in the force-fluctuated period observed reproducibly is probably the point at which the needle was inserted into a nucleus membrane. As shown in Fig. 4B, no such fluctuating curve was observed using a normal pyramidal AFM tip. Moreover, there was no difference between the DNA-immobilized nanoneedle and the non-immobilized one in the force curves of insertion processes into cells. This suggests that there is no specific interaction between DNA and cell components.

We calculated the friction force applied to the immobilized DNA in the penetration process through the cell membrane. The thickness of the cell membrane is about 7–10 nm, the contact area of nanoneedle and cell membrane is about 10,000 nm². The applied repulsive force to the nanoneedle is about 1 nN in the curve of Fig. 4A. So, the obtained friction force is about 0.1 pN/nm². The circumference of a double strand DNA molecule is about 40 nm, thus maximum contact area of DNA and cell membrane during penetration is about 400 nm². Therefore, the resulting friction force to one DNA molecule is about 40 pN, which is less than the unbinding force of 270 pN. This suggested that DNA on the surface of the nanoneedle was not detached by the friction force in the penetration step through the cell membrane.

The DNA-immobilized nanoneedle was observed with a confocal microscope (Fig. 5). The DNA immobilized on the nanoneedle was stained with 0.1 µg/ml TO-PRO-3 iodide, a monomeric cyanine nucleic acid stain. The whole surface of the DNA-immobilized nanoneedle and AFM cantilever was stained by the TO-PRO-3 iodide. The TO-PRO-3 iodide stained nanoneedle was inserted into a living cell 10 times. The manipulated cells after repeated insertions with a DNA-immobilized nanoneedle could proliferate normally, similar to non-manipulated cells. This suggested that the needle insertion did not cause critical damage to the manipulated cell.

After insertion, the nanoneedle was again observed by confocal microscopy in order to investigate DNA detachment from its surface. As shown in Fig. 6, it is obvious that all DNA molecules were not detached from the surface of the nanoneedle, though it is very difficult to estimate accurately the amount of DNA molecules released from the needle surface by confocal microscopic imaging alone. This result is consistent with the prediction from the friction force calculation.

We developed this system to deliver molecules into a living cell. In case of gene delivery, the goal is usually to maintain introduced DNA molecules in a nucleus. However, we aim to
introduce DNA molecules that can be removed from the cell after gene expression, i.e., not to make a recombinant cell. We are trying to develop a transient transcription technique of the DNA on the nanoneedle surface only during the inserting period. Thus, we do not expect any loss of delivered DNA until evacuation of the nanoneedle. All DNA was not detached from the surface of the needle in Fig. 6. We are also trying to develop the opposite goal of a controlled release of immobilized molecules from a nanoneedle surface.

4. Conclusions

We have successfully constructed a technique for molecular delivery into a living cell using nanoneedles. By monitoring force–distance curves we can confirm the immobilization of DNA on the surface of the nanoneedle and the insertion of the needle into a cell. All the immobilized DNA molecules on the needle surface were not detached from the needle in Fig. 6. We are also trying to develop the opposite goal of a controlled release of immobilized molecules from a nanoneedle surface.

Acknowledgement

This study was supported by the Industrial Technology Research Grant Program in 2003 from New Energy and Industrial Technology Development Organization (NEDO) of Japan. I. Obataya would like to acknowledge the financial support from NEDO Industrial Technology Fellowship Program.

References


