Cell Cultures Over Nanoneedle Fields

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Abstract

In this paper a new nanostructured support for the culture of cells is presented. The support consists of fields of sharp and high-aspect-ratio nanoneedles. The support is obtained through a specifically developed process that allows controlling the nanoneedles’ densities and height. The nanoneedles are typically 10 µm high with tip diameters under 200 nm. Cell viability on this support was evaluated through long-term cells cultures. The narrow interface between the cells’ membrane and the nanoneedles has been carefully observed to conclude on the perforation of the cells’ membrane thanks to the sharp nanoneedles. Such a nanostructured chip, allowing specific interaction, opens the door to a large number of exciting and valuable applications such as nanoporation for transfection or internal cell potential recording.

Key Words: Nanoneedles; field; cell culture; nanoscale membrane poration; silicon nanotechnology.

Introduction

Micro- and especially nanotechnologies are driving investigators to fabricate new culture supports with specific functionality for a large number of high-tech biotechnological applications such as gene transfection (1,2), electrofusion (3), high-throughput screening, and electrophysiology (patch-clamp and neuron activity recording) (4).

In this paper we present a silicon-based support with nanoneedle fields for practical applications at the cellular level. First, the nanostructured support is described with its specific useful geometries. Then the biological experiments performed with this support are detailed that demonstrate the biocompatibility of the support and its usefulness for biomedical sciences. Through the cells’ cultures on the support, the narrow interface between cells’ membrane and the nanoneedles is illustrated and discussed.

Material and Methods

Nanoneedles Field

The support is composed of nanoneedle fields. A specific microfabrication process was developed (patent pending) that allows forming nanoneedle fields out of silicon. This process of silicon etching RIE renders it possible to control the density and the height of the nanoneedles. Features obtained with this new microfabrication process allow at the same time high-aspect-ratio sharp needles and high-density needle fields contrary to a previous process that allow only sharp high-aspect-ratio single needles (5) with expensive and complex processes such as focused ion beam (6) or low-aspect-ratio needle fields (7).

The field density can vary between 4 and 40 nanoneedles/100 µm². Figure 1 illustrates two different nanoneedle field densities. The density of the field changes the interaction between the cell’s membrane and the nanoneedles.
The nanoneedle height can vary between 7 and 12 \( \mu \text{m} \). The nanoneedle tips are typically a few hundred nanometers (100–300 nm). The most interesting nanoneedle feature is its sharpness. Indeed, the aspect ratio of the nanoneedle is huge and the nanoneedles are sharply tapered as illustrated in Fig. 2, which shows a side view of nanoneedles in high- and low-density fields.

Cell cultures were grown on supports with various densities in order to characterize the biocompatibility and the biological impact of the interaction of the nanostructured needles and the cell’s membrane. The biological experiments are detailed in the next section, the culture and fixation protocols are explained, and observations are presented and discussed.

**Cell Culture on the Nanostructured Chip**

**Cell Culture**

Rat hepatoma (FAO) and human hepatoma (Huh7) cells (gift of C. Guguen-Guillouzo, INSERM U522, Rennes, France) were used. FAO cells were cultivated in a mixture of 50% HAM F12 medium (Sigma Aldrich) and 50% NCTC 135 medium (Eurobio) supplemented with 5% fetal bovine serum, 2 mM L-glutamine and 2 mL/L gentamycin (Sigma). Huh7 cells were cultivated in a mixture of 25% 199-2020 medium and 75% 2021 medium (Eurobio) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 5 \( \mu \)g/ml bovine insulin (Sigma), 0.5 \( \mu \)M hydrocortisone hemisuccinate, and 2 mL/L gentamycin. Cells were cultivated in 75 cm\(^2\) flask at 37°C in an humidified atmosphere of a 5% CO\(_2\) in air. Cells were pas-saged every week (1/2 dilution for FAO cells, and 1/6 dilu-tion for Huh7 cells) with trypsin–EDTA (Sigma); the medium was renewed every 2 or 3 d.

**FAO Cell Culture on Nanoneedles**

After trypsination, FAO cells were centrifuged for 5 min at 1500 rpm and resuspended in the culture medium. Three milliliters of FAO suspension were put on several nanoneedle chips (high and low nanoneedle density) in 30 mm Petri dish, with a common cell concentration of \( 1 \times 10^5 \) cell/mL (3 \( \times \) 10\(^5\) cells on a 700 mm\(^2\) surface corresponding to 400 cells/mm\(^2\)). Chips were kept at 37°C for culture and cell viability evaluation. Viability was monitored by cell proliferation after sev-eral culture days. Cell culture was stopped on the different chips after 1, 3, and 7 d. FAO cells were fixed with formalde-hyde 3.7% in PBS and dehydrated with increasing ethanol.

![Fig. 1. Nanoneedles field: (A) high nanoneedle density, (B) low nanoneedles density. Scale bar is 100 \( \mu \text{m} \).](image1)

![Fig. 2. Nanoneedles profile: (A) in a high-density field, 12 \( \mu \text{m} \) high nanoneedles with tips around 100 nm; (B) in a low-density field, 12 \( \mu \text{m} \) high sharp nanoneedles with tips smaller than 1 \( \mu \text{m} \) diameter.](image2)
concentrations (60% to 100%). Visualization was performed with a scanning electronic microscope (C.M.E.B.A., Rennes University, France).

Huh7 Cell Culture on Nanoneedles

After trypsination, Huh7 cells were centrifuged for 5 min at 1500 rpm and resuspended in the culture medium. Five milliliters of Huh7 suspension were put on several nanoneedle chips (high and low nanoneedle density) in 60 mm Petri dish, with a common cell concentration of $4 \times 10^5$ cells/mL ($2 \times 10^6$ Cells on a 2827 mm$^2$ surface corresponding to 700 cells/mm$^2$). Chips were kept at 37°C. Cell’s membrane portion was monitored by visualization of a fluorescent–labeled F-actin (Texas Red-X phalloidin, Molecular Probes), which is one of the cytoskeleton’s proteins and present at high concentration at the membrane vicinity for cytoskeleton anchoring purposes. Cell culture was stopped on the different chips after 1 and 3 d. Huh7 cells were fixed with formaldehyde 3.7% in PBS and labeled with Texas Red-X phalloidin (1/100 of phalloidin in PBS–Saponine 0.1%–SVF 1%). Labeled nanoneedle Huh7 cells were then mounted on glass coverslips and visualized with a confocal microscope (Rennes University, France). The confocal observations were made between the bottom of the nanoneedle’s support and the middle of the cell, corresponding to a height of 6.94 µm. For each observation (reflection and fluorescent), six planes were made, pictures are spaced out 1.15 µm.

Results and Discussion

Viability of Cells Cultured on Nanoneedle Chips

The cell viability was evaluated through its proliferation dynamic. After nanoneedle chip sowing with FAO cells and parallel culture at 37°C for several days, the culture was stopped on the different chips. After 1, 3, and 7 d, the cells were fixed and dehydrated. As shown in Fig. 3, the number of cells between d 1 and d 7 increases, whatever the nanoneedle density. This result shows that cells not only can live on this support nanostructured but that they can proliferate. The same observation was made with Huh7 cells (results not shown). Figure 3 illustrates the proliferation of the FAO between 24 h and 7 d.

Cell Membrane and Nanoneedle Interaction

Two sets of observation were undergone: SEM and confocal observations. SEM observations were made for sharp pictures and tilted visualization of membrane and needles. They are of great interest for high-density nanoneedles because, on those devices, reflection microscope observation is difficult due to light absorption. On the SEM pictures presented in the Fig. 4, we can observe a close contact between the cell’s membrane and the nanoneedles. During the fixation with formaldehyde and the dehydration steps, the cells shrink slightly and separation between cells parts of groups of cells were observed as illustrated in Fig. 4A. This indicates that the cells are immobilized by the nanoneedles. Close views of the membrane of the cells over the nanoneedles after the fixation and dehydration shrinkage (Fig. 5) reveal a membrane shape that indicates a retraction around the nanoneedles with the membrane partially retained by the nanoneedles. More striking pictures (Fig. 6) show membrane cracks due to shrinkage and the nanoneedle holding the cells membrane.

Nanoneedle density plays a crucial role on the way it interacts with cells. In the low-density case, cells get pierced, whereas in the high-density case, cells seem simply settle down over the nanoneedle tips. Our hypothesis is that the nanoneedles are thin and sharp enough to pierce the cell’s membrane under their own weight. The membrane is pierced and tightly surrounds the nanoneedles.

In order to test our hypothesis, confocal observations, for three-dimensional imaging of needles in the cell, were made. Cells were labeled with Texas Red-X phalloidin, a fluorescent dye of F-actin. Actin is one of the three main cell cytoskeleton components, which allow cells to have structure (shape and mobility). Although actin filaments spread about through the cell, they are mainly concentrated near the plasma membrane. Thus, membrane deformation can be characterized through actin deformation. Figure 7 shows one cell after 72 h culture over a low-density-nanoneedle field. Red fluorescence represents actin localization. Figures 7(A–C) show three planes of the cell with rising level, respectively, $z = 2.31$ µm, 3.47 µm and 4.63 µm. Figure 7(D)
shows a 3D reconstitution of the cell. At places where cell is penetrated by the nanoneedle, we can observe red bright circles (No. 2, No. 3, No. 4, No. 5) and spot (No. 1) of actin, showing the membrane surrounding the needle. Comparison of the localization of the red circles and spot with nanoneedles (Fig. 7E) shows that they are superimposable (Fig. 7F). Two possibilities are observed here. In case of No. 1, red spot is on the needle showing that the membrane is not pierced but raised by the needle. In case of No. 2, No. 3, No. 4, and No. 5, red circles are around the needles on several levels, without

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red spot on the highest planes (Fig. 7C for No. 3, figures not shown for No. 2, No. 4, and No. 5) showing that the membrane seems to be pierced.

The same observations were made with cells over low-density-nanoneedle fields after 24 h culture. In high density nanoneedles observations and conclusions are more complicated.
because light absorption makes visualization of the nanoneedles difficult.

Our hypothesis seems confirmed: nanoneedles seem to be thin and sharp enough to pierce the cell’s membrane, which then reseals around the nanoneedles.

Envisaged applications of such a support are numerous and exciting. The nanoneedle fields can be used for internal potential recording (8,9). For that purpose the needles have to be metallized and electrically connected as an array, hence enabling parallel recording of several cells’ internal potential.

Another application is the use of this nanoneedle array for the nanoporation as a means for protein, peptide, or genetic microinjection into living cells. Indeed, this kind of support could then be used for massive mechanical material transfer during the piercing of the membrane thanks to the nanoneedles. This could be a simple alternative to microinjection widely practiced using microcapillaries (10–12) that is often invasive due to shape and size of the microcapillaries or to the inaccuracy of their displacement.

Conclusion

A new and promising nanostructured support for cell culture and practical biomedical applications was presented. The support is made of arrays of silicon nanoneedles with controlled densities and height. The support was successfully evaluated as far as biocompatibility is concerned through long-term cultures that confirm cell viability and proliferation. SEM and confocal microscopy observations of the cell cultures over the supports reveal a tight interaction between the cell membranes and nanoneedles that opens the door to numerous and exciting biotech applications. Potential applications considered and future work cover internal neuronal cell recording for neural network studies and for mechanical DNA transfer for gene transfection.

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References