Cell spreading behaviors on hybrid nanopillar and nanohole arrays

Xiaomin Wu\textsuperscript{1,2}, Li Li\textsuperscript{1,2}, Lu Wang\textsuperscript{1,2}, Zecheng Lei\textsuperscript{1,2}, Fan Yang\textsuperscript{1,2}, Ri Liu\textsuperscript{1,2},
Ying Wang\textsuperscript{1,2}, Kuiqing Peng\textsuperscript{3}, and Zuobin Wang\textsuperscript{1,2,4}

\textsuperscript{1}Ministry of Education Key Laboratory for Cross-Scale Micro and Nano Manufacturing, Changchun University of Science and Technology, Changchun 130022, China
\textsuperscript{2}International Research Centre for Nano Handling and Manufacturing of China, Changchun University of Science and Technology, Changchun 130022, China
\textsuperscript{3}Beijing Key Laboratory of Energy Conversion and Storage Materials, Beijing Normal University, Beijing 100875, China
\textsuperscript{4}JR3CN & IRAC, University of Bedfordshire, Luton LU1 3JU, UK

E-mail: wangz@cust.edu.cn (Z. W.); lil@cust.edu.cn (L. L.); kq_peng@bnu.edu.cn (K. P.)

Received xxxxxx
Accepted for publication xxxxxx
Published xxxxxx

Abstract

Although nanopillars (NPs) provide a promising tool for capturing tumor cells, the effect of mixing NPs with other nanopatterns on cell behavior remains to be further studied. In this paper, a method of fabricating silicon nanoscale topographies by combining laser interference lithography (LIL) with metal assisted chemical etching (MACE) was introduced to investigate the behaviors and pseudopodia of A549 cells on the topologies. It was found that cells had a limited manner in spreading with small cell areas on the silicon nanopillar (SiNP) arrays, but a good manner in spreading with large cell areas on the silicon nanohole (SiNH) arrays. When on the hybrid SiNP/SiNH arrays, cells had medium cell areas and they arranged orderly along the boundaries of SiNPs and SiNHs, as well as 80\% of cells displayed a preference for SiNPs over SiNHs. Furthermore, the lamellipodia and filopodia are dominant in the hybrid SiNP/SiNH and SiNP arrays, respectively, both of them are dominant in the SiNH arrays. In addition, the atomic force acoustic microscopy (AFAM) was also employed to detect the subsurface features of samples. The results suggest that the hybrid SiNP/SiNH arrays have a targeted trap and elongation effect on cells. The findings provide a promising method in designing hybrid nanostructures for efficient tumor cell traps, as well as regulating the cell behaviors and pseudopodia.

Keywords: silicon nanopillar, silicon nanohole, cell spreading, subsurface features

1. Introduction

Circulating tumor cells (CTCs) are the general term for various types of tumor cells that shed from tumor lesions and then escape into the peripheral blood circulation [1]. These escaped tumor cells spread through the blood to different tissues of the body, causing tumor metastasis [2]. The capture and enrichment of tumor cells are of great significance for the early diagnosis and intervention of diseases, as well as the gene and protein analysis in the later stage. Nowadays, using topological structures to regulate cell behavior has become an important strategy for cell research in vitro [3-7]. Recently, nanostructures inspired by insect wings have been shown to be an effective physical strategy for cell capture, which kills cells by restricting cell activity and damaging cell membranes [8, 9]. Compared with conventional technologies, such as...
microchips and biomarkers [10-12], the biomimetic nanostructures have the advantages of simple preparation and high capture efficiency.

Great efforts have been made to investigate the capture of tumor cells from the aspects of preparation methods, substrate material selection and nano topography design. Some approaches for the fabrication of the bioinspired nanostructures have been carried out, such as electrodeposition [13], photolithography [14-16], reactive ion etching [17], and electron beam lithography [18]. However, these methods usually require harsh experimental conditions, complicated procedures, expensive equipment and even need to use harmful gases during the fabrication. For the selection of basement materials, silicon [19-21] and some soft materials with good biocompatibility and thermal stability [22-24], such as polylactic acid (PLA) [25], are preferred candidates. Currently, nanopillars have attracted great attention due to their high cell capture efficiency [26-28]. Compared with flat substrates, nanopillars provide a larger effective contact area for antibody-receptor binding, thus improving cell capture efficiency [1]. The isolation and capture of tumor cells were studied by adjusting the diameter, height and spacing of nanopillars. Moreover, other nanostructures, such as nanowires, nanotubes [29, 30], and functionalized surfaces, such as those modified with cancer cell capture agents [31], have also attracted attention in the field of tumor cells capture. However, the preferred nano topography for cell capture and specificity is still unclear. In view of the fact that the nanostructures used for the capture of tumor cells are generally a single type of nanostructures, and different nanostructures have different effects on cells. For instance, nanopillars facilitate cell capture but restrict cell spreading, while nanopores are beneficial to cell spreading but have relatively low cell capture efficiency. Therefore, the strategy of mixing nanopillars with other nanostructures is an attractive topic, and the efficiency of the hybrid nanostructures on cell capture is worthy of further study. Studying the mechanism of interaction between cells and nano topologies is necessary for designing optimal nanostructures for cell capture.

The simple and convenient methods for fabricating nanostructures, as well as the mechanism of interaction between cells and nano topologies are both important in exploring how cells respond to the surrounding environment. In this study, we investigated the effect of nano topographies on cell behavior and pseudopodium by culturing A549 cells on SiNP arrays, SiNH arrays and hybrid SiNP/SiNH arrays. Specifically, the three nano-patterned structures were fabricated by combining LIL with MACE. After 24 hours incubation on the three nano topographies, the scanning electron microscopy (SEM) was applied to investigate cell behaviors. In addition, the atomic force acoustic microscopy (AFAM) was performed to detect the subsurface features.

2. Methods

2.1 Fabrication of hybrid Si nanostructures

The hybrid Si nanostructures were fabricated by laser interference lithography (LIL) combined with metal-assisted etching (MACE) [32-34]. In brief, the polished P(100) silicon wafers were cut into 1 cm × 1 cm pieces, and then were ultrasonically degreased with acetone, ethanol and deionized water. The Ag film of 15 nm thick was coated on the cleaned silicon surface after they were treated with oxygen plasma for about 10 minutes. The laser interference system containing Nd: YAG laser (INNOLAS, 1064 nm wavelength, 8 ns pulse, and Gaussian beam) and two-beam interference system was used to obtain Ag nanopatterns with the period of 14 μm, and the single beam energy ranged from 40 mJ to 65 mJ. After these steps, the silicon wafers with Ag nanopatterns were immersed into etching solutions (HF and H₂O₂, 10% and 0.6% v/v, respectively) for 45 minutes. In order to avoid the influence of Ag particles on cell experiments, the corroded samples were then immersed in HNO₃ with the concentration 65%~68% for 30 minutes. In addition, all the nanopatterned substrates were soaked in deionized water overnight to remove the residual corrosive solution before cellular experiments.

2.2 Cell culture

A549 cells, belong to the human lung adenocarcinoma cell line, were used in our research. Before plating cells, the nanopatterned substrates and silicon substrates (control group) were sterilized under ultraviolet lamp for 1 hour, and then rinsed three times with the phosphate-buffered saline (PBS) solution. Cells were maintained in the RPMI-1640 medium supplemented with 1% penicillin-streptomycin (Solarbio) and 10% fatal bovine serum (FBS) at 37°C, 5% CO₂ humidified incubator.

2.3 Atomic force microscopy (AFM) characterization of substrates

An atomic force microscope (JPK, Nano Wizard 3, Germany) performed in the tapping mode was used for the measurement of the topography and surface roughness of the substrates. A rotated monolithic silicon Tap300Al-G probe (Budget Sensor, China) with the nominal spring constant of 40 N/m and free resonance frequency of 300 kHz was used. The topographic images of substrates were captured at the drive frequency of 265.381 kHz and the reference point value of 0.5 V.

2.4 Cell fixation

To characterize the morphology of A549 cells incubated on the hybrid SiNP/SiNH arrays by SEM (FEI: Quanta 250 FEI), cell fixing in advance was necessary. The cells with the density of 160 × 10⁴ cells mL⁻¹ were added to a 24 well-plate with the substrates placed in the wells. After 24 hours
and 72 hours incubation, the cells were rinsed with PBS three times and then fixed in the 4% glutaraldehyde solution overnight (4°C), followed by again rinse with PBS. Afterwards, cells were dehydrated in ethanol at the concentrations of 50%, 70%, 80%, 90%, 95% and 100%, further dehydrated in tert-butanol overnight (-20°C) and finally dried in the vacuum oven. At last, a gold film of 5nm thick was deposited on the cells.

2.5 Immunofluorescence

After 24 hours of incubation, samples were treated by 4% paraformaldehyde solution for about 10 minutes, followed by 5% Triton X-100 treatment for 5 minutes to increase cell membrane permeability. After the steps, the samples were stained with FITC-Phalloidin (Solarbio) for 30 minutes. Subsequently, the samples were incubated with 4',6-diamidino-2-phenylindole (DAPI) (Solarbio) for 5 minutes. In the experiment, the samples were rinsed two or three times for 10 minutes by PBS in each staining step. Fluorescence images were captured using an inverted microscope with the objective magnification of 40× (DS-Ri2, Nikon, Japan).

2.6 Cells viability

The cell viability was tested by MTT assay. Cells with the density of $5 \times 10^4$ cells mL$^{-1}$ were added to a 24 well-plate with the substrates placed in the wells. One well with the cell suspension (density of $5 \times 10^4$ cells mL$^{-1}$) added was acted as the control group. After 12 hour and 24 hour incubation, 100 μL of MTT solution (5 mg/mL in PBS) were added into each well, followed by 37°C incubation for 4 hours. Then, 500 μL of DMSO was added into each well, followed by shocking for 10 minutes. Finally, 500 μL of mixture was added into a 96 well-plate, and the absorbance was read by a microplate reader at the wavelength of 490 nm.

2.7 Quantification of cells

Image J software was used to quantify the cell length and area. The Feret’s diameter that the longest distance of two points on the cell boundary was defined as the cell length. 50 cells ($0.8 \times 0.5$ mm$^2$ area) were selected from each of the experimental groups (hybrid SiNP/SiNH, SiNP and SiNH areas) and the control group (Si wafer) to measure the cell
lengths. 30 cells were randomly selected from the SEM images of various types of substrates to measure the average cell areas. SEM images were also selected to quantify the ratio of cells on the nanopillars in the hybrid SiNP/SiNH areas.

2.8 Atomic force acoustic microscopy (AFAM) characterization of cells

Before AFAM characterization, cell fixation was carried out, which was the same as the cell solidification method presented in the "Cell fixation" section. AFAM (CSPM5500, Benyuan, China) was performed to detect the subsurface features. Vaseline was applied on the back of the sample to make it in close contact with the sample stage to reduce the signal attenuation. The ACCESS-FM probe (APPNANO, USA) with the spring constant of 2.7 N/m was used to the subsurface features test with the resonant frequency of 38.414 kHz and the reference point value of 0.1 V. The collected data were processed by Imager 4.60 (Benyuan, China).

3. Results and Discussion

Fig. 1(a) shows the complex Ag patterns fabricated by laser interference lithography (LIL). The Ag nanoparticles (AgNPs) formed in the areas of maximum intensity distributions, while the Ag film appeared in the areas of minimum intensity distributions [35, 36]. Fig. 1(b) presents the corresponding Si nanostructures of Fig. 1(a) after 45 minutes etching by 10% HF and 0.6% H$_2$O$_2$ (v/v) solution, followed by 65%–68% HNO$_3$ treatment for 30 minutes. The formations of hybrid Si nanopillar and Si nanohole (hybrid SiNP/SiNH) arrays are due to the effect of metal assisted chemical etching (MACE). During the etching process, Ag generates electron holes by reducing H$_2$O$_2$ [32]. The electron holes are injected into the Si at the Ag/Si interface and cause the oxidation of the Si to be dissolved by HF. As time goes by, the Si is continuously etched downward to form the silicon nanoholes. Finally, the areas where AgNPs exist eventually form Si nanopores (SiNPs), while the areas where the silver film exists form Si nanopillars (SiNPs). Fig. 1(c) shows the corresponding cross-sectional profiles of Fig. 1(b), and the enlarged SEM images of SiNPs and SiNHs are shown in the blue dashed box and red dashed box, respectively. It can be seen that the top of the SiNPs are relatively flat, similar to nails with unsharp tips. The etching paths of SiNPs are perpendicular to the surface, while the etching paths of SiNHs are mainly perpendicular to the surface but with some twisting. Those twisty SiNHs show the "walking trace" of AgNP during the sinking [37], resulting in a shallower depth of SiNHs compared to SiNPs, as shown in Fig. 1(d). The average heights/depths of SiNPs and SiNHs are about 1.6 μm and 1.3 μm, respectively. The detailed geometrical properties of pillars and holes are shown in Table 1 (Supporting information). Note that there is no AgNP at the bottoms of SiNHs and SiNPs, which proves the effectiveness of HNO$_3$ to treat the substrate after etching to avoid the influence of Ag on cell experiments.

To better understand the characteristics of the substrate, the topography and surface roughness of the hybrid SiNP/SiNH arrays were examined by AFM, as shown in Fig. 2. The AFM topographic image of the hybrid SiNP/SiNH arrays in Fig. 2(a) matches its SEM image in Fig. 1(b). In the AFM image, the bright fringes correspond to the SiNHs, while the dark fringes correspond to the SiNPs. The mean surface roughness values of nanohole (NH) area and nanopillar (NP) area are about 2.32 nm and 10.03 nm, respectively. The relative height between NHs and NPs in the hybrid SiNP/SiNH array marked with the white line in Fig. 2(a) is shown in Fig. 2(b). From the cross-section curves, it can be clearly seen that the topography of NH arrays is about 20 nm higher than that of NP arrays. The corresponding 3D image of hybrid SiNP/SiNH array shown in Fig. 2(c) presents that the NHs (dark fringes) are higher than the NPs (bright fringes), which further confirms the view exhibiting in Fig. 2(b). The AFM images of SiNPs and SiNHs are also presented in Fig. S1 (Supporting information).

The viabilities of cells cultured on Si, SiNP, SiNH and hybrid SiNP/SiNH substrates for 12 hours and 24 hours were test by MTT assay, as shown in Fig. S2 (Supporting information). The results showed that the cell viabilities increased after they were cultured for 12 hours and 24 hours on the substrates, which indicated that the substrates had good biocompatibility [13, 14]. Specifically, cell viabilities on the Si, SiNH and hybrid SiNP/SiNH substrates were similar to
that on the control group, while the cell viability on the SiNP substrate is higher than those on the other substrates. The results that SiNPs promote cell proliferation are consistent with those of other nanopillars [1, 25].

To study the morphology and spreading of cells on these types of arrays, the topography images of cells were characterized by SEM. Fig. 3(a) and Fig. 3(b) show the SEM images of A549 cells cultured on the control group (Si wafer)
and experimental groups (SiNP, SiNH and hybrid SiNP/SiNH arrays) after 24 hours incubation, respectively. The cells on the Si wafer spread well and radially in all directions, and the lamellipodia and filopodia are clearly presented. While on the experimental group, the spreading profile of cells cultured on the SiNP array is different from that on the Si wafer. The cells become round and the pseudopodia extend out in a limited manner, resulting in a relatively smaller cell size. In contrast, the cells incubated on the SiNH arrays stretch out as freely as those on the Si wafer with long and multiple filopodia. For the cells cultured on the hybrid SiNP/SiNH arrays, they stretch and align along the direction of nanohole arrays. The high magnification SEM image on the right further shows the details of cell elongation and spreading, from which it can be clearly seen that the cells take the edge of the nanohole array as the grip, and extend along the pattern into a spindle shape.
The SEM images of A549 cells cultured for 72 hours on the substrates are also presented in Fig. S3 (Supporting information). Compared with Fig. 3 (24 hours), the cells on the SiNP arrays have a larger spreading area and the filopodia disappear, while the cells on other substrates have no significant difference. The corresponding fluorescence microscope images of the cells cultured on the Si, SiNP, SiNH and SiNP/SiNH for 24 hours are shown in Fig. S4 (Supporting information). The cell behaviors shown in the fluorescence images are consistent with those shown in the SEM images.

It is clearly presented in Fig. 3 that there have obvious differences in the morphology and spreading of cells on the different arrays. In order to describe these phenomena more accurately, the quantitative characterization of cell length and area were performed. As shown in Fig. 4(a), the cells on the hybrid SiNP/SiNH arrays have the longest cell length of about 44 μm, and the cells on the Si wafers and SiNH arrays have the almost same cell lengths with the mean values of 34 μm and 35 μm, respectively, while the cells on the SiNP arrays have the shortest cell length about 26 μm. The results are consistent with the phenomenon of cell spreading presented in Fig. 3. On the other hand, the mean areas of cells on these arrays were also quantitatively analysed. The results in Fig. 4(b) show that the mean areas of cells on the SiNH arrays (416 μm²) and Si wafer (371 μm²) are greater than those on the hybrid SiNP/SiNH (271 μm²) and SiNP arrays (204 μm²). Among them, the cell mean area on the Si wafer is smaller than that on the SiNH arrays, but the cell mean area on the hybrid SiNP/SiNH is larger than that on the SiNP arrays.

From the above qualitative (Fig. 3) and quantitative (Fig. 4) analyses, the influences of different arrays on cell behavior can be concluded. Generally, as an important substrate material for cell research in vitro, Si wafer has biocompatibility. Cells on the SiNH arrays present almost the same cell lengths and larger cell areas compared with those on the Si wafer, indicating that the SiNH arrays facilitate cell spreading [1, 13]. Nanopores increase the effective contact area between the cells and the substrate without hindering cell spreading, which has been confirmed by previous studies [38]. When on the SiNP arrays, cells have the shortest cell length and smallest spreading area, which is due to the fact that NPs are similar to unsharp nails and act as hindrances to the cell spreading process [9, 39]. The results suggest that SiNP arrays have a negative effect on cell spreading. While on the hybrid SiNP/SiNH arrays, cells have the longest cell length and larger cell area compared with those on the SiNP arrays. The results indicate that mixing the SiNH with the SiNP arrays can improve the cell spreading, and the topographic guidance provided by the boundary between the SiNP and SiNH makes the direction of cell arrangement consistent with the grating direction.

**Figure 6.** (a) SEM image of A549 cells cultured on the SiNP/SiNH arrays for 24 hours. (b–c) Corresponding details of cells of (a). (d) Histogram depicts the percentage of cells on the NP and NH measured from the cells on the hybrid SiNP/SiNH arrays. ***p < 0.001, t-test. It is noted that the data are shown as the mean ± standard errors.
To further investigate the influence of the SiNP and SiNH arrays on cell behavior, the details of cell spreading were presented. The SEM images in Figs. 5(a-b) show the details of pseudopodia in different areas of the same cell (on the bottom left Fig. 5(a)) cultured on the SiNP arrays. Similarly, the details of pseudopodia in different areas of the same cell (on the bottom left Fig. 5(c)) cultured on the SiNH arrays are shown in Figs. 5(c-d). The inset images on the bottom left of (a) and (c) with the scale bars of 10 µm show the full views of cells in a lower magnification. The images with the scale bars of 4 µm show the details of cells in a higher magnification. On the SiNP arrays, the lamellipodia only exist near the cell around with the restricted spreading and the filopodia are more frequently observed with shorter lengths compared with those on the SiNH arrays. While on the SiNH arrays, the lamellipodia and filopodia are dominant and they spread freely with clear edges. Besides the pseudopodia, stress fibre bundles (as shown in Fig. 5(d)) also appear at the edge of the cell, which are not observed in the cells cultured on the SiNP arrays. Study has shown that lamellipodia are dominant when the adhesion force between the substrate and cell is relatively large, while filopodia are dominant when that adhesion force is relatively small [1]. Therefore, the adhesion force of cells on the SiNP arrays is smaller than that on the SiNH arrays in this study.

Similarly, cell behavior on hybrid SiNP/SiNH arrays is also worthy of further investigation. Fig. 6 presents the details of cells cultured on the SiNP/SiNH arrays. It is clearly shown in Fig. 6(a) that the direction of cell is consistent with the grating, and the cell extends along the boundary of SiNP and SiNH arrays. The spreading of cells is larger than that on the SiNP arrays, which suggests that mixing SiNH with the SiNP arrays can improve cell spreading. In addition, the lamellipodia are more frequently observed in the array. As mentioned above, the adhesion force is large when lamellipodia are dominant but it is small when filopodia are dominant. Therefore, it is reasonable to infer that the cell adhesion force on SiNP/SiNH arrays is larger than that on the SiNP arrays. Fig. 6(b) shows the corresponding detail of cell marked with the red box in Fig. 6(a). The stress fibres with obvious directionality on the lamellipodia are in the same direction as the grating. This kind of directional spreading only appears on the SiNP/SiNH arrays, while the spreading of lamellipodia on the SiNP or SiNH arrays is radial in all directions. Fig. 6(c) exhibits the corresponding detail of cell marked with blue box in Fig. 6(a). It shows the edge of the lamellipodium, which is located on the SiNPs. In order to better understand the growth behavior of cells on the hybrid SiNP/SiNH arrays, the ratio of cells on the nanopillars (NPs) located on the surface of the hybrid SiNP/SiNH arrays is also quantified, as shown in the Fig. 6(d). The observations show that, almost 80% cells on the NPs, but only 20% on the nanoholes (NHs). This may be due to the fact that the nanopillars similar to the unsharp nails capture the cells that fall on them and restrict their movement, which is consistent with the previously report that the nanopillars have a trapping effect on cells [9, 17].

Atomic force acoustic microscopy (AFAM) can obtain the information of subsurface by distinguishing the stiffness of

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**Figure 7.** (a) AFAM topographic image of A549 cells on the SiNP arrays for 24 hours. (b) The details of cells in the red box with a high magnification of (a). (c) The corresponding acoustic image of (a). (d) The details of cells in the red box with a high magnification of (c). It is noted that the yellow circles mark the different regions between (b) and (d).
different materials, which cannot be obtained in the above SEM images. However, studies have shown that the thickness of samples is the major factor to influence the subsurface detection [40]. The limit range of AFAM detection is generally less than 1 μm, and the centre height of the cell is generally a few micrometres [33], so the edge of the cell is selected to observe the subsurface structure. As shown in Fig. 7, the A549 cells cultured on the hybrid SiNP/SiNH arrays after 24 hours incubation was imaged by the AFAM. The topography image (Fig. 7(a)) does not show a clear vision of the cell edge and nano-pattern substrate, and the interface between the cells and SiNHs is not clearly presented in its corresponding high magnification image (Fig. 7(b), marked with yellow circles). In contrast, the corresponding acoustic image in Fig. 7(c) clearly presents the cell morphology and substrate pattern, meanwhile, the SiNPs buried in the cell edge are clearly presented in yellow circles in Fig. 7(d). Hence, the subsurface information provided by AFAM acoustic image is able to make up for the lack of topography images, providing accurate cell topography information.

4. Conclusions

In this work, the silicon nanoscale topographies fabricated by LIL combining with MACE were proposed to reveal their effects on A549 cells. The experiment results showed that the SiNP arrays restricted the cell spreading and the filopodia were clearly observed. The SiNH arrays promoted the cell spreading, and the lamellipodia and filopodia were also clearly observed. However, on the hybrid SiNP/SiNH arrays, the cells were trapped and extended directionally. Specifically, about 80% of the cells were selectively grown on the SiNP and the lamellipodia were dominant, as well as the cells were elongated along the boundaries of SiNP and SiNH arrays. In addition, the subsurface features of samples were also revealed by AFAM images. The findings provide a promising method in designing hybrid nanostructures for efficient tumor cell traps, as well as regulating the cell behavior and pseudopodia.

Acknowledgements

This work was supported by National Key R&D Program of China (No.2017YFE0112100), EU H2020 Program (MNR4SCOLL No.734174), Jilin Provincial Science and Technology Program (Nos.20180414002GH, 20180414081GH, 20180520203JH, 20190201287JC, 201907202002GH and 20200901011SF), “111” Project of China (D17017), and National Natural Science Foundation of China (51972031). This work was also partially supported by Changli Nano Biotechnology (China).

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