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Dense diamond nanoneedle arrays for enhanced intracellular delivery of drug molecules to cell lines

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Abstract Nanotechnologies for intracellular delivery are of great value in clinical and biological research. Diamond nanoneedle arrays are a novel and attractive platform to facilitate drug delivery with minimal cytotoxicity. Using our technique, the cellular membranes can be temporarily disrupted for enhanced diffusion of drug molecules to cytoplasm. Herein we show that this technique is applicable to deliver different types of anticancer drugs into a variety of cell lines, although the membrane of each cell line possesses varied rigidity and hardness and each drug has its own unique properties and targets. When anticancer drugs and nanoneedle arrays are collaboratively used to treat cancer cells, the cell viability dramatically decreases by up to 40 % in comparison with the cells treated with drugs only. Attractively, therapeutic molecules can be efficiently delivered to drug-resistant cells with the aid of nanoneedle arrays. The combination of diamond nanoneedle arrays and anticancer drug cisplatin can decrease the viability of A549 cisplatin-resistant cells to about 60 %, while the cells only treated with the same concentration of drug are essentially not affected due to their drug resistance. These results indicate that dense nanoneedle arrays represent an effective approach to enhance the delivery of biological molecules to different types of cells. Such approach will certainly be beneficial to microbiological research and clinical applications in the future.

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Introduction

Intracellular delivery is of great importance in not only basic research but also clinical applications. During last decades, a great variety of biological, chemical, and physical approaches have been or are being developed for delivering different materials and molecules to cells [1–3]. Among them, physical approaches are now receiving tremendous interest due to their unique characteristics. In a study, Kim et al. demonstrated that living cells can be cultured on the surface of silicon nanowires and the nanowires are able to naturally penetrate into cells within a period of incubation time [4]. During this process, the pre-coated DNA on the silicon nanowires can be delivered to the cells and is capable of transfection in vivo. Similar approaches are also tested for transporting a series of biomolecules to cells [5].

Although effective, in these methods, cells are cultured on the tips of nanostructures (e.g., silicon nanowire arrays and aluminum oxide nanostraws [4–9]), and therefore the surface area of the nanoneedles substrate will limit the maximum number of cells in the treatment. It also takes a

comparatively long period of time to culture and penetrate cells, so the efficiency of this approach is expected to be low. Additionally, it is unknown how the transfected cells are available for further applications and whether the nanostructures will cause any effect to the cells if they are remained within the interior for a long period of time. Therefore, there is an uprising demand to further improve the technique for increasing efficiency, shortening time required for drug delivery process and minimizing potential effect to cells.

To meet these requirements, we recently fabricated diamond nanostructure arrays and applied cell suspensions to the nanostructures for improved intracellular delivery [10–13]. With choice of diamond as the material, our nanoneedles are robust enough to breach the membranes. Through the cell suspension treatment approach, it is convenient to process a large number of cells at one time without facing the issue of a patch having a limited area and the treatment time is very short. Herein, we further explore whether this method can be used for effective delivery of drugs to a series types of cancer cells, particularly to drug resistance cells. In this study, we use nanoneedle arrays to deliver two different anticancer drugs to four types of cell lines and study their viabilities under various conditions, through which we expect to promote the nanoneedle arrays-based platform for intracellular delivery studies.

Materials and methods

Materials

SN38 and cisplatin were purchased from Acros Organics. DMSO was ordered from Sigma. DMEM, FBS, penicillin–streptomycin, PBS, and MTT were obtained from Invitrogen.

Fabrication of sharp diamond nanoneedle arrays

Diamond nanoneedle arrays were fabricated according to a previously reported method [14–17]. Briefly, diamond nanoneedle arrays were fabricated in a plasma CVD reactor. Firstly, an n-type (100)-oriented 1–10 Ω silicon wafer polished with diamond particle paste (0.25 μm) was used as a substrate. The ASTeX MWCVD reactor connected with a 1.5 kW microwave generator was accounted for deposition of polycrystalline diamond film. The MWCVD reactor was heated up to about 800 °C with 1200 W microwave power, while the mixture of H_2/CH_4 plasma with the ratio of 99 %/1 % was pumped into the MWCVD reactor. About 10 μm thick polycrystalline diamond film was then deposited onto the substrate.

Subsequently, the film was placed into an ECR-MPCVD. A magnetic field of 875 G generated by an eternal magnetic coil and plasma generated by an ASTeX 1.5 kW microwave were applied to the reaction chamber. A mixture of $He/Ar/N_2$ gases with the ratio of 70 %/25 %/5 % was used to produce the plasma. The chamber temperature and pressure were kept at 400 °C and 1.0×10^{-3} Torr, respectively, for 1–8 h to produce nanoneedle arrays with different configurations. The morphology of the nanoneedle arrays was observed by scanning electron microscopy (SEM, XL 30).

Cell culture

MC-3T3, NIH-3T3, HeLa, and A549 cells were cultured at 37 °C with 5 % CO_2 and 95 % humid air in DMEM (Gibco) containing 10 % FBS (Gibco). The medium was renewed every 2 days and confluent cells were subcultured by trypsin.

A549-CR cisplatin-resistance cells (A549-CR) were cultured in full culture medium containing 0.5 μg/ml cisplatin for the first screening. Then the remaining cells were cultured in full culture medium containing 1.0 μg/ml of cisplatin for 8 weeks.

Application of diamond nanoneedle arrays

Cells were trypsinized and suspended in DMEM. Then the cells were counted and diluted. The cell suspension for experiments contains about 1×10^4 cells per milliliter. Two anticancer drugs including SN38 and cisplatin were added into cell suspensions at specific concentrations.

Before cell treatment, diamond nanoneedle arrays were sterilized by 70 % ethanol for at least 30 min. The arrays were then washed with PBS (phosphate-buffered saline) for three times to remove all ethanol. Subsequently, 1 ml of cell suspension with or without drugs was flushed toward the diamond nanoneedles rapidly, and this procedure was repeated for 20 times by a 1-ml pipette. The control experiment of applying cells to a smooth Si wafer was also conducted with the same procedures. After treatment, the cells were incubated in media in the presence or absence of drugs for 30 min. Next, for the cells incubated in a medium containing drug, centrifugation was employed to remove drug and fresh DMEM medium was added. Finally, the cells were planted into a 96-well plate and cultured for 24 h for microscopy and MTT analysis.

MTT assay

5 mg/ml of MTT was prepared in PBS and stored at −20 °C. 10 μl of the MTT solution were added to each

well of a 96-well plate and the final concentration was 0.5 mg/ml. Then the plate was incubated at 37 °C for 4 h. Finally, 100 µl of DMSO was added into each well and mixed to ensure complete dissolution of formazan crystals. Finally, the absorbance at 540 nm of each sample was measured.

Quantity analysis of intracellular delivery of cisplatin into cells

In the presence of 5 µg/ml cisplatin, A549 cell suspension was treated with diamond nanoneedle arrays for intracellular delivery. During treatment, 1 ml of suspension contained about 200,000 cells. After treatment, the cells were incubated at 37 °C for 30 min. The control group was A549 cells which were incubated with the same concentration of cisplatin for 30 min, but without nanoneedle treatment. Subsequently, the A549 cells were collected by centrifugation at 800 rpm for 5 min, and washed twice with cold PBS to remove extra cisplatin and resuspended in PBS. The cell number was analysed with hemocytometer. Then a suspension was prepared with a concentration of 10^7 cells in 1 ml of PBS. Next, the cells were centrifuged at 800 rpm and cell pellet was collected for Pt content determination. A volume of 400 µl 65 % HNO_3 was added to the pellet and incubated overnight at 65 °C for releasing intracellular Pt. 600 µl Milli Q water was added into the lysate to a final volume of 1 ml. The Pt content was ascertained by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES, PE-2100DV).

Statistical analysis

The experiments were repeated for at least three times. For each set of experiments, the result was expressed as mean values ± standard deviations. Statistical significance was calculated by either the Student *t* test for paired comparisons or the one-way ANOVA for multiple comparisons. A p value <0.05 was considered significant.

Results

SEM characterization of diamond nanoneedle arrays

Figure 1 shows a typical SEM image of dense diamond nanoneedles with heights varied from 200 nm to 1.3 µm. The nanoneedles are very sharp with tip radius of about 10 nm, allowing them to easily penetrate the cell membranes.

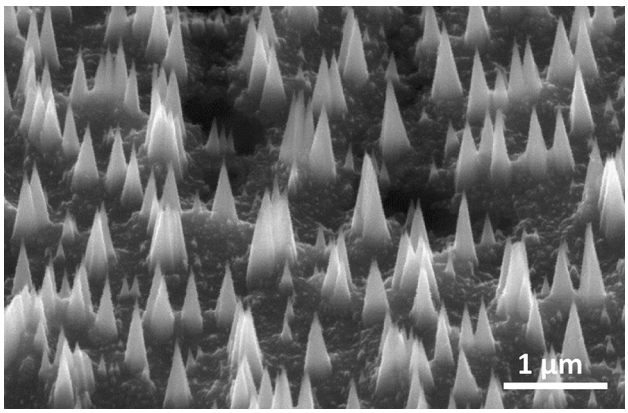

Fig. 1 Scanning electron microscopy (SEM) images of diamond nanoneedle arrays. The *scale bar* indicates 1 µm

Effect of nanoneedle treatment on cells

Before investigating the intracellular drug delivery with nanoneedle array treatment, it is necessary to study its potential effect on cells. Three groups were investigated: control group (untreated cells), cells treated with smooth silicon substrate, and cells treated with sharp nanoneedles. Optical microscopy and MTT test were conducted to observe the cell morphology and quantify the cell viabilities after different treatments.

Figure 2 shows the optical images of NIH-3T3 cells that are not treated and treated with either smooth silicon substrates or sharp nanoneedle arrays. In terms of cell morphology, no obvious difference is observed among the three groups. Therefore, we can deduce that the cell morphology is not noticeably affected by treatment. Qualitatively, from these optical microscopy images, no obvious cell death was found after treatment.

To quantitatively confirm that the nanoneedle treatment does not cause cell death, we conducted an MTT test of untreated cells and the cells treated with sharp nanoneedle arrays or smooth silicon substrates. The results are shown in Fig. 3. The cell viability of untreated cells is normalized to 100 %. It is clear that there is negligible difference of viabilities among these three groups, suggesting that nanoneedle treatment should not lead to significant cell death. Based on these data, it can be expected that such nanoneedle array treatment may act as a low-toxic technique for enhanced intracellular delivery.

Delivery of drug molecules to different cell lines

After confirming that the cell morphology and viability are harmlessly affected by diamond nanoneedles treatment, we explored the application for enhanced intracellular delivery of an anticancer drug by quantifying the viability of cells.

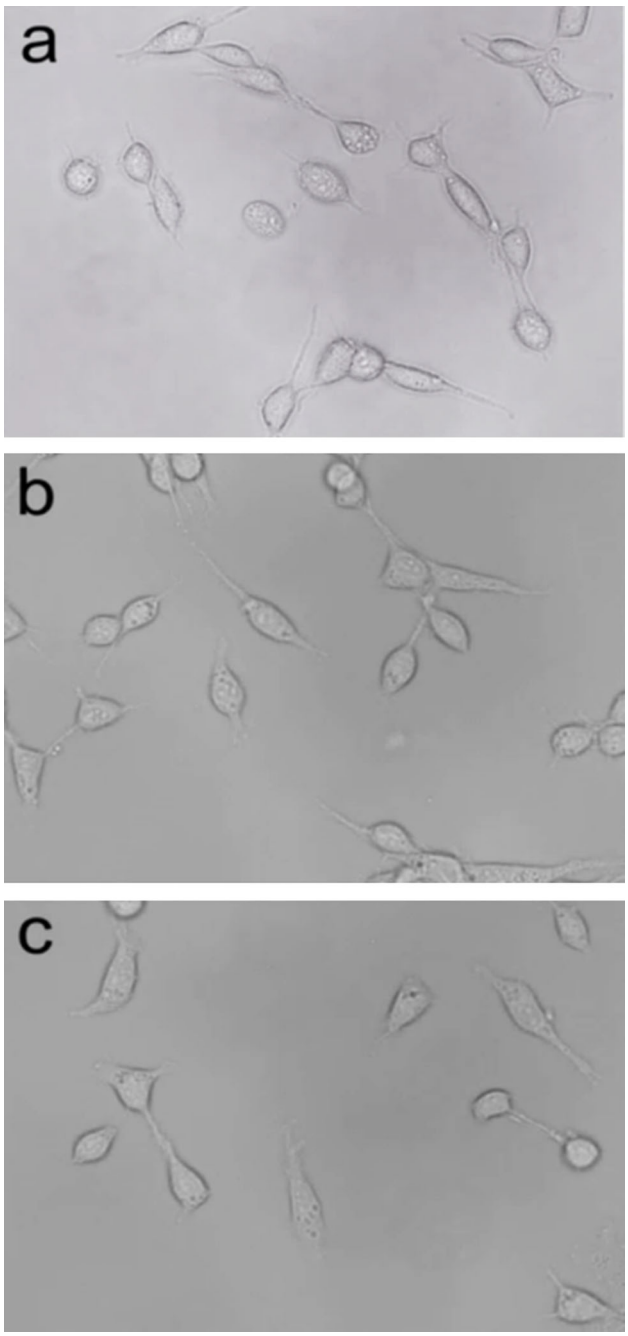

Fig. 2 Optical microscopy images of NIH-3T3 cells: **a** 6 h after being treated with smooth silicon substrate, **b** 6 h after being treated with diamond nanoneedle array, and **c** with no treatment

Broad spectrum anticancer drug SN38 is widely used in cancer treatment, so we choose it as one test case. To investigate if the technology can be universally used for a range of cells, four different cell lines including MC-3T3, NIH-3T3, HeLa, and A549 cells were selected. In the test, 2 μg/ml of SN38 was added into cell suspensions. Since the viabilities of untreated cells and the cells with smooth Si substrate treatment do not have statistical difference

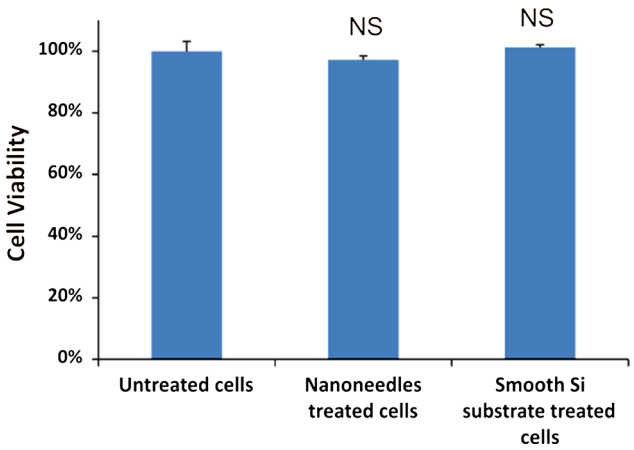

Fig. 3 Viability of NIH-3T3 cells after being treated with smooth silicon substrates and diamond nanoneedle arrays. Untreated cells were used as a control group. Data were analysed for significance using ANOVA test, followed by *t* test for multiple comparisons. NS: $p > 0.05$

(Fig. 3), we used untreated cells as a control group in the following experiments.

The results are normalized in relation to the number of cells in the control group of untreated cells. Comparing with the control group, the viabilities of cells treated with nanoneedles in all groups decrease about 20 % (Fig. 4).

Intracellular delivery of different drugs

After demonstrating nanoneedle array treatment can facilitate intracellular delivery of drugs to various cell lines, we also investigated if this technique can be used for different drug molecules. For this purpose, SN38 and cisplatin were chosen and the results are shown in Figs. 5 and 6. From the figure, it can be seen that, at all concentrations and for different cell lines and drugs, the viabilities of nanoneedle treated cells are always lower. For SN38, the cell viabilities are 10–35 % lower in nanoneedles treated groups compared with control groups at different drug concentrations among all four cell lines (Fig. 5). While with cisplatin treatment, the cell viabilities drop by up to nearly 40 % when we compare the nanoneedles treated groups with the control group among all cell lines (Fig. 6). Furthermore, we also quantitatively measured the intracellular delivery of cisplatin in A549 cells by ICP-OES. In the presence of 5 μg/ml of cisplatin, A549 cell suspension was treated with or without diamond nanoneedle arrays. The uptake of Pt in the cells without diamond nanoneedle array treatment is 15.3 ng of Pt per 10^7 cells, while the level increases to 103.7 ng of Pt per 10^7 cells when the cells are treated with diamond nanoneedle arrays. This clearly demonstrates that diamond nanoneedle arrays are capable to transport drug molecules into cells with enhanced delivery efficiency.

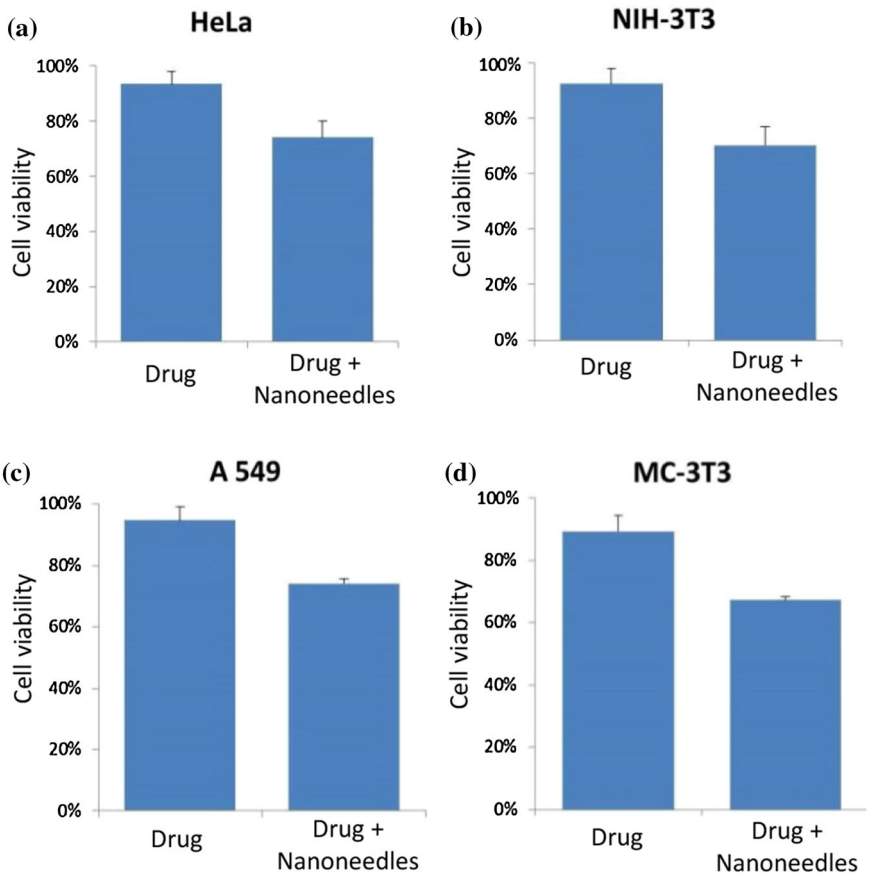

Fig. 4 Viabilities of four cell lines: **a** HeLa, **b** NIH-3T3, **c** A549, and **d** MC-3T3 cells. For each cell line, one group of cells was incubated with 2 μg/ml of SN38, but without nanoneedle array treatment; the other group of cells was treated by both nanoneedle arrays and 2 μg/ml of SN38. For all groups, after 30 min incubation with SN38, the medium in each well was replaced by fresh one, and then the cells were incubated in the fresh medium without drugs for 24 h. Untreated cells were used as a negative control group and the viability value was normalized as 1

Intracellular delivery to A549 cisplatin-resistant cells

Since diamond nanoneedle arrays have already been proved to be an efficient tool to deliver different drugs into a range of cell lines, we expect that diamond nanoneedle arrays can also enhance the delivery of cisplatin to drug-resistant cells and increase the effectiveness of the drug to kill the cells. To test our hypothesis, human lung carcinoma A549 cells were chosen to establish cisplatin-resistant (CR) cells. The parental cisplatin-sensitive A549 cells were exposed to 1 μg/ml cisplatin for 8 weeks until the resistant index of the A549-CR cells was found to be over 20-fold higher than that of the parental A549 cells.

The IC_{50} of A549 cells treated with cisplatin is 1 μg/ml, but for A549 cells with resistance to cisplatin, this dose is even not enough to induce noticeable cell death. Three groups of A549-CR cells were conducted in this experiment. The untreated group of A549-CR cells cultured in the absence of 1 μg/ml cisplatin was used as negative control. The second group of A549-CR cells was only treated with cisplatin and the third group was treated by both cisplatin and nanoneedle arrays. Both groups were cultured in the presence of 1 μg/ml of cisplatin for 48 h. Then the viability was tested by MTT assay. As shown in Fig. 7, the treatment with 1 μg/ml of cisplatin does not lead to noticeable cell death, but with the aid of nanoneedle array treatment, with the same concentration of cisplatin, the viability of the A549-CR cells dramatically decreases to about 60 %. This strongly proves that the nanoneedle array treatment can successfully enhance the drug delivery process even for drug resistance cancer cells.

Discussion

In the present study, we used nanoneedles arrays to mechanically disrupt cell membranes for increasing the delivery of two different drugs into four types of cells. It is expected that nanoneedle array treatment only causes temporarily disruption of the cell membrane of a variety of cells despite their different mechanical properties. Through this, intracellular delivery can take place directly in an effective way and lead to enhanced therapeutic action and high efficacy of the drugs.

Although a number of materials such as Si, SiO_2, Al_2O_3, and carbon fibers have been previously used to make nanostructure arrays for intracellular delivery, we choose diamond because of its superior mechanical properties and

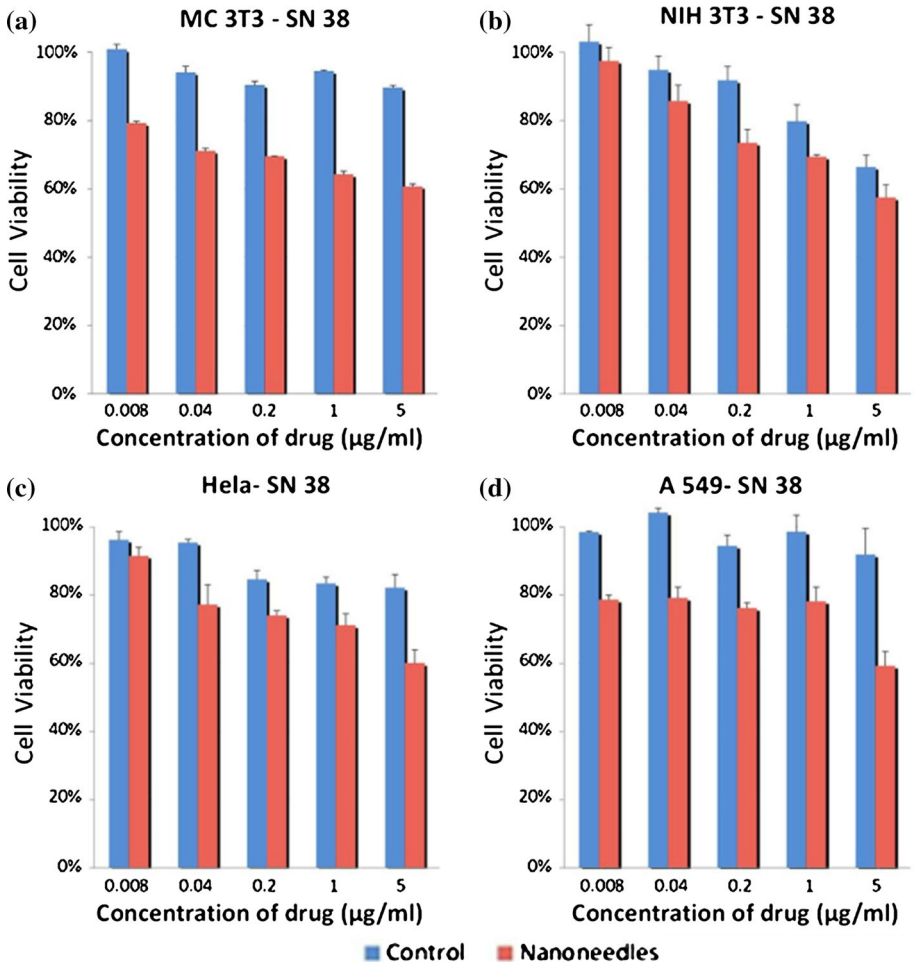

Fig. 5 Viabilities of four cell lines: **a** MC-3T3, **b** NIH-3T3, **c** HeLa, and **d** A549 cells. For each cell line, one group of the cells was only treated with SN38 and the other group treated by both SN38 and nanoneedle arrays. For all groups, after 30 min incubation with SN38, the medium in each well was replaced by fresh one and then the cells were incubated in the fresh medium without drugs for 24 h. Untreated cells were used as a negative control group and the viability value was normalized as 1. The concentrations of SN38 used in the test were 0.008, 0.04, 0.2, 1, and 5 μg/ml

chemical inertness [4–17]. In our assay, we flush cell suspensions to densely packed nanoneedles and expect to achieve a high efficiency to treat a large amount of cells at once. Thus, our treatment approach is high-throughput comparing with an individual nanoneedle system and others' reported approaches of culturing cells on nanostructure arrays for passive penetration. In addition, the nanoneedle treatment in our method is temporary. The contact time between nanoneedle tips and cells is extremely short. Once the intracellular delivery process is complete, the cells can be freely used for further studies, applications, or characterizations, without being affected by the nanostructure arrays. In these aspects, our approach is superior to the previously reported technologies involving culturing cells on nanostructure arrays for intracellular delivery.

In our test, we performed the treatment of using diamond nanoneedle arrays to cells followed by observation of the cell morphology and quantitative cell viability analysis. The results indicate that the cell morphology does not show noticeable difference from that of the untreated cells and the cell viability has no statistical difference from that of the untreated cells. As the nanoneedles are extremely densely packed and the length is also very short, we would not expect that the nanoneedles can penetrate through the cell membranes. Instead, we believe that these findings suggest that the nanoneedles should only gently disrupt the membranes for enhanced molecule diffusion and the disruptions of nanoneedle arrays to cells should be reversible and the treated cells be able to recover automatically.

Once we confirm that the technique does not lead to noticeable cell death, we tested the delivery of anticancer drugs to four different cell lines including MC-3T3, NIH-3T3, HeLa, and A549. In our test, SN38 and cisplatin were chosen as these two anticancer drugs have broad spectrum of activity but distinct working mechanisms. SN38 is the active metabolite of irinotecan that inhibits topoisomerase I [18]. Cisplatin is one of the most commonly used and clinically successful anticancer drugs [19, 20]. Series of platinum-based compounds have been synthesized and evaluated as potential anticancer drugs [21]. From our results, it is obvious that, with nanoneedle array treatment and for the same concentration of SN38 and cisplatin, the cytotoxicity is dramatically improved in all of the four test cell lines, although the cell membranes of different cell

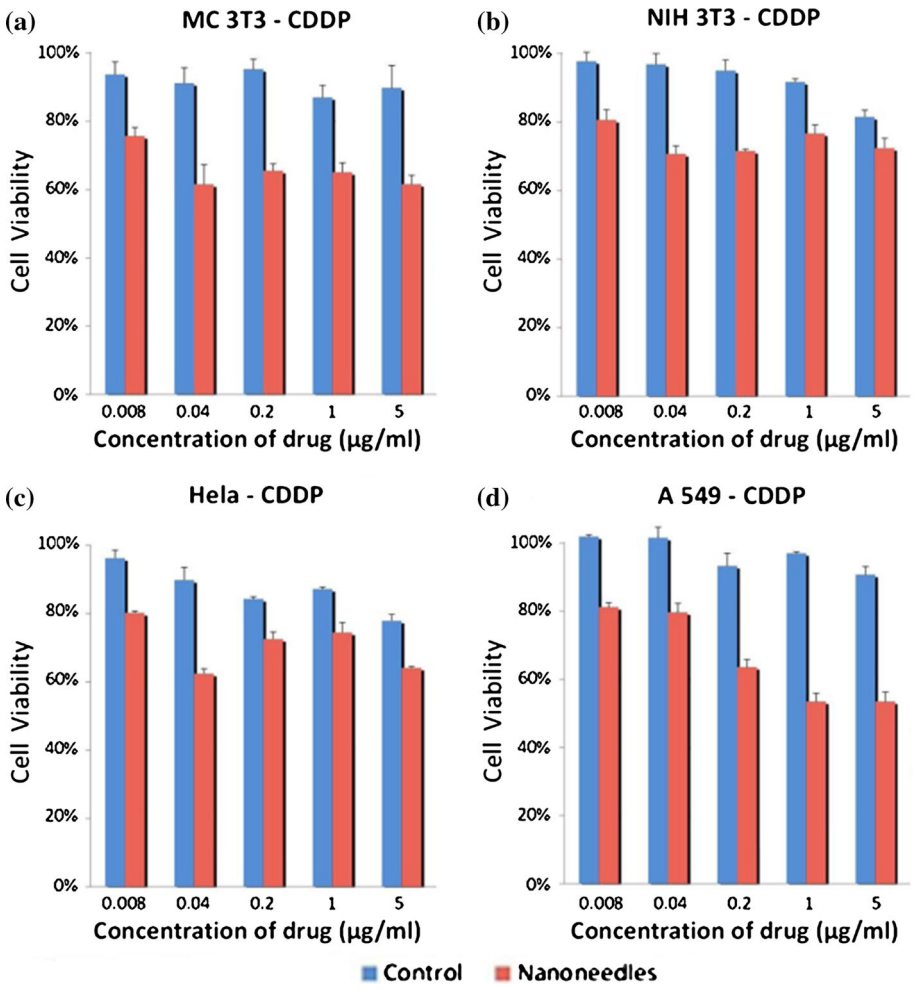

Fig. 6 Viabilities of four cell lines: **a** MC-3T3, **b** NIH-3T3, **c** HeLa, and **d** A549 cells. For each cell line, one group of the cells was only treated with cisplatin and the other group treated by both cisplatin and nanoneedle arrays. For all groups, after 30 min incubation with cisplatin, the medium in each well was replaced by fresh one, and then the cells were incubated in the fresh medium without drugs for 24 h. Untreated cells were used as a negative control group and the viability value was normalized as 1. The concentrations of cisplatin used in the test were 0.008, 0.04, 0.2, 1, and 5 μg/ml

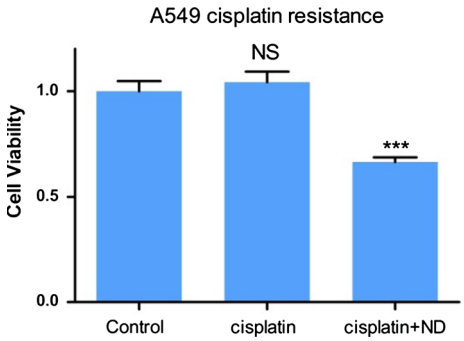

Fig. 7 Viability of A549 cisplatin-resistant (A549-CR) cells. One group of A549-CR cells was only treated with cisplatin and the other group treated by both cisplatin and nanoneedle arrays. Both groups were cultured in the presence of 1 μg/ml cisplatin for 48 h. Untreated cells were used as a negative control group, and the viability value was normalized as 1. NS, $p > 0.05$; ***$p < 0.001$

lines possess different rigidity, Young's modulus, and hardness [22, 23]. For example, the Young's modulus of HeLa cells and 3T3 fibroblasts are 1.8 and 12 kPa, respectively, when measured by an AFM probe. This indicates that the physical treatment of diamond nanoneedle arrays to cells is likely to be effective to a wide variety of cells. Our results also suggest that the nanoneedles system be not limited to specific cell lines, which offers board potential applications for the treatment.

After we show that our diamond nanoneedle array technology can significantly enhance the delivery of anticancer drug molecules to cells and lead to increased cancer cell death, we expect that such a technology may be applied to treat drug-resistant cancer cells. With the improved intracellular delivery, a relatively high dose of drugs will be transported into cells and it might be beneficial to overcoming drug resistance. Indeed, an attractive perspective is that the nanoneedles drug delivery system is proved to be a very easy and effective method in enhancing cisplatin delivery into A549 cisplatin resistant cells (Fig. 6). This again shows that the technique is able to deliver drug molecules to a broad range of cell lines and has the potential to be a universal and efficient in vitro intracellular delivery tool.

Conclusions

Novel, highly dense, and sharp diamond nanoneedle arrays are fabricated by microwave plasma CVD. These nanoneedle arrays are able to treat a large number of cells for significantly enhanced drug delivery. This approach is applicable to different drugs and various types of cells. Particularly, it is intriguing that the method can facilitate the delivery of molecules to drug resistance cells for greatly improved therapeutic efficacy.

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