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### **Diamond-Nanoneedle-Array-Facilitated Intracellular Delivery and the Potential Influence on Cell Physiology**

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5 **The potential influence of using diamond nanoneedle arrays for intracellular delivery on**  
6 **cell physiology**

7

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33 **Keywords:** Diamond nanoneedle arrays; intracellular delivery; reactive oxygen species;  
34 glucose 6-phosphate dehydrogenase; mitochondrial membrane potential

35

36 **Abstract:** Vertical arrays of nanostructures can direct access to cell cytoplasm and probe

37 intracellular molecules. Here, we report an efficient cytosolic delivering platform by simply

38 combining diamond nanoneedle arrays with centrifugation-induced supergravity. We prove

39 that diamond nanoneedle arrays can efficiently deliver drugs and biomaterials into cytosol

40 within several minutes, negotiating the endocytosomal system on cytomembrane. More

41 importantly, we studied the potential influence of the technique on cell metabolism. By

42 detecting the phosphorylated histone variant H2AX (pH2AX) in the nucleus, we prove that

43 the operating process will not lead to DNA double-strand breaks. However, the mechanical

1 disruption can temporarily improve the permeability of the cell membranes. Furthermore, we  
2 find that nanoneedle treatment can affect the metabolism in living cells. The treatment can  
3 slightly elevate the apoptotic signal in A549 cells. The diamond nanoneedle array treatment  
4 can significantly increase the production of reactive oxygen species (ROS) in cells,  
5 particularly when working together with anticancer drug molecules. Meanwhile, the activity  
6 of cytosolic glucose 6-phosphate dehydrogenase (G6PD) is also raised to counterbalance the  
7 elevated ROS content. The result of the marked depolarization of mitochondrial membrane  
8 potential suggests that mitochondria have involved in the intracellular redox reactions and  
9 cell apoptosis, which are induced by diamond nanoneedle treatment. Overall, we  
10 systematically investigate the potential influence of diamond nanoneedle arrays treatment on  
11 the physiology of cells and this study provides a novel understanding on the intracellular  
12 delivery mediated by nanoneedles.

13

## 14 **1. Introduction**

15 Delivery of biological molecules and fluorescent probes into living cells and tissues is of great  
16 importance.<sup>[1,2]</sup> A broad range of cationic polymers, lipids, transfection and complex-  
17 assembling reagents, as well as nanomaterials have been widely used in cytosolic delivery.<sup>[3-11]</sup>  
18 However, many factors affect the efficiency of cargo molecules delivery, such as cell type,  
19 cell number, media, carrier-relating cytotoxicity and low transfection efficacy for hard  
20 transfecting cells.<sup>[12,13]</sup> Recently, as a physical approach, vertical nanostructure arrays are  
21 reported to conveniently facilitate intracellular delivery with high throughput.<sup>[14]</sup> For example,  
22 vertical silicon nanowires have been developed as a universal platform for transporting a  
23 range of cargos into living cells, including DNAs, RNAs, peptides and proteins.<sup>[3,15]</sup> In the  
24 delivering process, cells are cultured on the vertical nanostructures for a period of time so that  
25 the nanowires spontaneously penetrate cell's membranes and the pre-coated biomolecules on  
26 the nanostructures can be slowly released into the cells. Similarly, aluminium oxide

1 nanostraws have also been applied to deliver cargos into cells but the difference is that the  
2 nanostraws are hollow and integrated with a reservoir so materials can be directly injected to  
3 cells once the nanostraws pierce into cells during the culturing.<sup>[16]</sup> The advantage of these  
4 methods is that biomolecules can be transported into cells without the requirement of  
5 chemical modification or viral packaging. However, some cell functions might be perturbed  
6 by culturing on such nanostructures for extended period of time. For example, it has been  
7 discovered that HeLa cells grow slowly<sup>[17]</sup> and temporarily develop irregular contours when  
8 culturing on silicon nanowires and the penetration of nanowires into cells leads to lipid  
9 scrambling although this can be reversed in healthy cells.<sup>[15]</sup> In addition, Persson *et al.*  
10 explored that fibroblasts cultured on similar nanostructures display impaired cell division,  
11 increased reactive oxygen species (ROS) content and ultimately DNA damage.<sup>[18]</sup> Liu *et al.*  
12 found that mesenchymal stem cells exhibit significantly different adhesion, proliferation and  
13 differentiation behavior when compared to those on flat substrates.<sup>[19]</sup>

14 To alleviate these problems, we developed diamond nanoneedle arrays for enhanced  
15 intracellular delivery.<sup>[20-22]</sup> In our approach, instead of culturing cells on the vertical  
16 nanostructures for spontaneous penetration over a period, we allow the nanoneedles to  
17 actively disrupt the cell membranes within a very short duration (e.g., seconds to minutes) by  
18 a precisely controlled centrifugation force or applying a cell suspension to the nanostructures  
19 with a certain speed.<sup>[20-22]</sup> This platform has been demonstrated capable of effectively  
20 delivering fluorescent cell probes (iridium(III) polypyridine complex, FITC-dextran,  
21 propidium iodide (PI), and calcein AM, semiconductor quantum dots), drug molecules  
22 (cisplatin), and genes (siRNA and DNA) to various cell lines.<sup>[20,21,23]</sup> Very recently, with a  
23 similar manner, carbon nanosyringe arrays have also been employed to facilitate the delivery  
24 of bioactive molecules to hard-to-transfect cells.<sup>[24]</sup> Beyond these *in vitro* uses on cell lines,  
25 biodegradable silicon nanoneedles with 50-nm-diameter tips are applied *in vivo* to transfect  
26 VEGF-165 gene and induce sustained neovascularization.<sup>[25]</sup> This work sets a new milestone

1 in the application of nanoneedle arrays for delivering biological functional molecules to the  
2 cells in living tissues.

3 Despite the promising future of this new approach, the effect of the method on cell  
4 physiology has not been investigated although it is of great importance to the design of the  
5 nanostructures and operation parameters. In this study, we apply cells in a suspension to  
6 diamond nanoneedle arrays with centrifugation force for efficient intracellular delivery and  
7 then investigate the potential influence of the method on cell's DNA, ROS content,  
8 mitochondrial membrane potential and glucose-6-phosphate dehydrogenase (G6PD) activity.  
9 These findings will certainly improve our understanding of the technology of using this type  
10 of vertical nanostructures for intracellular delivery and should provide guidance to better  
11 design the platform for practical applications.

12

## 13 **2. Results and discussion**

### 14 **2.1. Fabrication of diamond nanoneedle arrays**

15 Diamond nanoneedle arrays were fabricated by forming a polycrystalline diamond thin film  
16 first on silicon substrates followed by microwave plasma etching. We specifically chose  
17 diamond to manufacture nanoneedle arrays because of its outstanding mechanical strength  
18 and inertness. Two representative SEM images of diamond nanoneedle arrays are shown in  
19 **Figure 1a**. These nanoneedles are conical in shape with height of 2  $\mu\text{m}$  and density of  $1 \times 10^6$   
20  $\text{mm}^{-2}$ . The tip of diamond nanoneedle arrays is about 50 nm.

### 21 **2.2. Diamond nanoneedle arrays to facilitate intracellular delivery**

22 In our previous reports<sup>[20,22]</sup>, we used diamond nanoneedle arrays to improve intracellular  
23 delivery by applying a cell suspension to the nanostructures with a certain speed. The method  
24 is high-throughput and effective but lack of precise control. To further improve this approach,  
25 herein, we apply a cell suspension on a diamond nanoneedle arrays and then apply

1 centrifugation force to carefully control the interaction between cells and nanostructures for  
2 enhanced intracellular delivery (Figure 1b). At the end of the process, the cell suspension is  
3 collected.

4 Before measuring the potential influence of the technology on cell physiology, we firstly  
5 proved that this system could be used to rapidly transport fluorescent molecules into cells. In  
6 our experiment, we chose FITC labelled dextran with large molecular weight of 10,000  
7 Daltons for this evaluation and the results are presented in **Figure 2a**. Three groups were  
8 performed including untreated cells, cells incubated with FITC-dextran without nanoneedles  
9 treatment, and cells treated with both nanoneedles and FITC-dextran. From the figures, it can  
10 be observed that the untreated cells and the cells incubated with FITC-Dextran for minutes  
11 but without nanoneedles treatment display no or negligible fluorescence. In great contrast,  
12 FITC-dextran is able to quickly transport into the nanoneedles treated A549 cells within only  
13 5 minutes. Calculated by the software Image J, the relative fluorescence intensities in the cells  
14 without and with diamond nanoneedle application are 10% and 57%, respectively (Figure 2b).

15 After confirming that the method can effectively deliver fluorescent molecules into cells,  
16 we then investigated whether this method was capable of improving the delivery of  
17 biologically functional small molecules to cells. Tests on three groups were conducted. In one  
18 group,  $0.2 \mu\text{g ml}^{-1}$  of cisplatin was added into cell suspension and then the cells were placed  
19 on a diamond nanoneedle substrate followed by application of centrifugation force of 30  
20 seconds only. Following that, the treated cells were cultured for 48 hours for viability  
21 measurement. Another group was A549 cells in a suspension containing the same  
22 concentration of cisplatin but without nanoneedle treatment. Untreated A549 cells were used  
23 as negative control group. The cell viability of all three groups was measured 48 hours later.  
24 The results were normalized to the number of cells in the negative control group. Compared  
25 with the negative control group, there was no reduction of cell viability observed in the group  
26 of cells treated with cisplatin but without nanoneedle treatment. It indicates that the

1 concentration of cisplatin is very low and not enough to kill cells. However, with additional  
2 nanoneedle treatment, the viability of cells dramatically drops by 32% in the presence of the  
3 same concentration of cisplatin (Figure 2c). Considering that the duration of nanoneedle  
4 treatment time might affect the drug delivery efficiency, we tested the cell viability by  
5 increasing the centrifugation time from 30 seconds to 5 minutes. As shown in Figure 2d, the  
6 viability of cells decreases over 60%. Overall, the results in Figure 2 demonstrate that this  
7 method is efficient in intracellular delivery.

8 Since the nanoneedle platform has been proved to be an efficient system in cytosolic  
9 delivery, we also expect that diamond nanoneedle arrays can enhance the delivery of drugs  
10 into drug resistant cells. We tested the potential efficiency of this system in doxorubicin  
11 resistant MCF7 cell line (MCF7/ADR) by flow cytometry. Three groups were conducted in  
12 the experiment. The first group of untreated MCF7/ADR cells was used as a negative control  
13 group. The second group of MCF7/ADR cells was incubated with  $2 \mu\text{g ml}^{-1}$  doxorubicin for 5  
14 minutes. The third group of cells was treated by both nanoneedles and doxorubicin at the  
15 same time. The results are described in Figure 2e. It can be seen that the efficiency of  
16 MCF7/ADR doxorubicin delivery in the diamond nanoneedle arrays group (27.53%) is higher  
17 than that in the doxorubicin free-delivery group (17.11%). The fluorescent intensity of  
18 doxorubicin in control group is only 5.6%. Furthermore, we also evaluated the use of  
19 nanoneedle treatment in helping doxorubicin to kill MCF7/ADR cells. When MCF7 cells are  
20 cultured with  $1 \mu\text{g ml}^{-1}$  doxorubicin for 48 hours, the cell viability is only about 10% (Figure  
21 S1, Supporting Information). In comparison, for MCF7/ADR cells, this dose is even not  
22 strong enough to induce significant cell death. Attractively, with the aid of diamond  
23 nanoneedle arrays treatment, the viability of MCF7/ADR cells can be reduced to about 50%  
24 (Figure 2f) in the presence of the same concentration of doxorubicin. Collectively, these  
25 results suggest that diamond nanoneedle arrays be an efficient delivering platform even for  
26 drug resistant cells.

### 1 2.3. Toxicity of diamond nanoneedle array treatment

2 While confirming that diamond nanoneedle treatment is able to facilitate the transport of  
3 molecules into cells and improve the efficacy of drugs, the next key question is whether the  
4 methodology leads to cytotoxicity.

5 To investigate if our intracellular delivery approach has any impact on cell proliferating  
6 ability, we firstly performed a viability study of cells. One group of untreated cells was used  
7 as a control group. The other group of cells was treated with diamond nanoneedle arrays by  
8 centrifugation at 120 g for 5 min at room temperature. The total number of cells at the  
9 beginning of the experiments in each group is about 30,000. Then the cell viabilities of both  
10 groups of cells were determined with an MTT assay and the results are shown in **Figure 3a**. It  
11 is clear that the diamond nanoneedle arrays treatment does not cause measurable cell death in  
12 comparison with the untreated cells.

13 Following cytotoxicity measurement, to find out whether our approach induces DNA  
14 damage, we investigated the integrity of genomic DNA in cells. One response to double  
15 strand DNA break is the need of phosphorylated histone variant H2AX (pH2AX) in the  
16 recruitment of proteins responsible for DNA repairing.<sup>[26, 27]</sup> In other words, the pH2AX  
17 indicates DNA double-strand breaks. Therefore, we measured the level of DNA damage by  
18 specific antibody-based detection of pH2AX in nucleus. In the test, cells were treated by  
19 diamond nanoneedle arrays followed by culturing in a 96-well plate for 3, 6 and 24 hours.  
20 Untreated cells were again used as a negative control group. Then all of the groups of cells  
21 with different culturing times were labelled with pH2AX mouse monoclonal antibody.  
22 Subsequently, a fluorescent secondary antibody was used to bind to the primary pH2AX  
23 antibody and the fluorescence intensities were determined for quantity analysis of pH2AX.  
24 As shown in Figure 3b, the amount of pH2AX does not have significant difference between  
25 the control group and the group treated by nanoneedle arrays at all 3 different time points.



1 This indicates that the diamond nanoneedle array treatment does not lead to DNA double-  
2 strand breaks.

3 Besides genotoxicity, we also measured the integrity of cytoplasm membrane at 3, 6, and  
4 24 hours after diamond nanoneedle array treatment with the image-iT dead green viability  
5 stain. We previously demonstrated that diamond nanoneedle disruption to cells can lead to the  
6 permeation of ethidium homodimer-1 (EthD-1) through the cell membranes.<sup>[21]</sup> EthD-1 is a  
7 high-affinity nucleic acid stain that is weakly fluorescent until bound to DNA and emits red  
8 fluorescence. In the test, EthD-1 was applied when cells were undergoing nanoneedle  
9 treatment. However, here, we add Image-iT dead green at 3, 6, and 24 hours after nanoneedle  
10 treatment. Image-iT dead green staining is a microscopy-based fluorescent assay for dead  
11 cells. It is impermeable to health cells but become permeant when the cytoplasm membrane is  
12 compromised. The result about image-iT dead green staining is presented in Figure 3c. It is  
13 clear that there is negligible difference of fluorescence intensity between the untreated cells  
14 and the group treated by nanoneedle arrays at different time points, suggesting that the cell  
15 membranes recover their integrity soon after nanoneedle disruption (Figure 3c).

16 We next monitored the release of glucose 6-phosphate dehydrogenase (G6PD) from  
17 treated cells into the surrounding medium. G6PD is a cytosolic enzyme and can leak from  
18 dead cells. The released amount of G6PD in cell medium correlates with the number of dead  
19 cells in the sample. Since the level of G6PD activity in common cell culture sera is much  
20 lower than many other enzymes, such as lactate dehydrogenase, this assay can be more  
21 precise and effective. A549 cells were treated with a diamond nanoneedle arrays following by  
22 24 hours culturing in a 96-well plate. The extracellular G6PD level was detected by following  
23 the vibrant cytotoxicity assay kit. The mechanism is to oxidise glucose 6-phosphate by G6PD  
24 and generate NADPH, which cause the reduction of resazurin (a blue dye) to yield fluorescent  
25 resorufin for measurement. Cell culture medium and untreated cells were used as two control  
26 groups. The result shows that there is no significant difference among these three groups in

1 the amount of extracellular G6PD (Figure 3d). These findings suggest that diamond  
2 nanoneedle treatment delivering system not affect the viability of the cells. This is in line with  
3 the result shown in Figure 3a.

#### 4 **2.4. Nanoneedle treatment leads to apoptosis**

5 After confirming that diamond nanoneedle treatment does not cause measurable necrosis and  
6 DNA damage and the cell membrane integration at 3 hours after treatment is also not affected,  
7 we then investigate whether the nanoneedle will lead to cell apoptosis and influence the  
8 intracellular G6PD level.

9 For cell apoptosis measurement, A549 cells were treated with a diamond nanoneedle  
10 array followed by culturing for 24 hours. Subsequently, the cells were tested by alexa fluor  
11 488 annexin V/dead cell apoptosis kit. Annexin V was used as a probe to monitor apoptotic  
12 signals. In apoptosis, phosphatidylserine is exposed to the external cellular environment  
13 through lipid scrambling and annexin V can specifically bind to phosphatidylserine for  
14 detection of apoptosis of living cells. Red-fluorescent PI was used to stain cells in the early  
15 stage of necrosis by permeating the cell membranes and binding to nucleic acid within cells.  
16 Similar with the results shown in Figure 3, it can be seen from **Figure 4a** and **4b** that diamond  
17 nanoneedle treatment alone does not induce notable cell death. Comparing to the control  
18 group (Figure 4a) (2.21%), the population of early necrotic cells in the groups of cells treated  
19 by a diamond nanoneedle array were low (1.57% in Figure 4b (cells were treated by a  
20 diamond nanoneedle array alone); 0.71% in Figure 4d (cells were treated by a diamond  
21 nanoneedle array and 5  $\mu\text{g ml}^{-1}$  cisplatin). However, the total population of apoptotic A549  
22 cells in the nanoneedle treated group slightly increases from 1.31% to 3.17% (Figure 4a, b).  
23 Furthermore, when A549 cells are incubated with 5  $\mu\text{g ml}^{-1}$  of cisplatin, the population of  
24 apoptotic A549 cells is 11.54%. When the cells are treated by nanoneedles and cisplatin at  
25 the same time, the population of apoptotic cells will increase to 16.59%. This also indicates

1 the drug efficacy of cisplatin is enhanced through the aid of nanoneedle treatment. In brief,  
2 the results show that nanoneedle treatment slightly induces cell apoptosis.

### 3 **2.5. The recovery of mechanical disruption on the permeability of cells**

4 The enhanced intracellular delivery results (Figure 2), together with the apoptotic experiments  
5 (Figure 4) suggest the disruption on the cell membrane induced by nanoneedle treatment. In  
6 the meantime, the toxicity test (Figure 3) proves the integrity of the cytomembrane after  
7 diamond nanoneedle treatment. Therefore, it is useful to investigate the recovery process of  
8 the cell membrane after being treated with nanoneedles. This will also provide guidance on  
9 the application of the device.

10 For this purpose, calcein-AM and PI were chosen to stain the treated cells. Calcein-AM is  
11 highly lipophilic and cell membrane permeable dye which can generate calcein from calcein-  
12 AM by esterase in a living cell and emit green fluorescence (maximum excitation: 490 nm,  
13 emission: 515 nm). Therefore, calcein-AM can only stain living cells. PI is not permeant to  
14 living cells, and therefore has been commonly used to detect the cells with comprised  
15 membranes such as dead cells. To understand the recovery of the cell membrane after  
16 nanoneedle disruption, calcein-AM and PI were added into the medium of the treated cells at  
17 different time points. The group without diamond nanoneedle treatment was set as a control  
18 group. In the control group, a certain number of calcein-AM molecules can enter the cells, but  
19 nearly no PI is able to diffuse to the cells because of the integrity of the cell membranes.  
20 Comparing with the control group, the cytosolic delivery of calcein-AM is dramatically  
21 enhanced when it was added immediately (0 min) after the cells being treated by nanoneedles  
22 (**Figure 5a**). This is within our expectation as the diamond nanoneedle treatment is supposed  
23 to improve the permeability of the cell membrane. With the increase of the time, the cell  
24 membrane should gradually recover and therefore the permeation of molecules into the cells  
25 should slowly drop. After a certain period of time, the cell membrane is expected to  
26 completely recover, so the permeation of PI molecules into cells should not be possible any

1 more. In line with this theory, we indeed find that the fluorescence intensity of the cells has  
2 the highest value at 0 min. When calcein AM was added at 5, 10 and 20 mins after the cells  
3 being treated by nanoneedles, the fluorescence intensities slightly drop because fewer  
4 molecules are able to enter the cells. When calcein AM was added at 40 mins or later, the  
5 fluorescence intensities fall to the level which is consistent with that of the control group  
6 (Figure 5a, b). This means that the cell membrane should have recovered its integrity. These  
7 findings are in fact fully supported by the PI staining result. For example, when PI was added  
8 to the medium at 40 mins or later, negligible PI molecules can enter the cells, so the  
9 fluorescence can hardly be observed in the cells. We also calculate the percentages of PI  
10 positive cells at each time point. The results indicate that the percentage of PI positive cells  
11 are all above 90% within the 20 minutes period after nanoneedle treatment, but the value  
12 dramatically drops to negligible amount (control level) at 40 minutes. Taking together, we  
13 suggest that diamond nanoneedle arrays penetration indeed disrupts cell membrane, but the  
14 integrity of the cell membrane can recover within 40 minutes of nanoneedle treatment  
15 gradually.

## 16 **2.6. Nanoneedle treatment produces high content of ROS**

17 In order to examine the ROS content in cells, we used CellROX Green reagent. The dye is  
18 weakly fluorescent in a reduced state but displays high intensity of green fluorescence when  
19 oxidized by ROS. Untreated cells without staining were used as a negative control. The ROS  
20 content of cells was tested at two time points (12 and 24 hours after treatment) under different  
21 conditions. As shown in **Figure 6a**, after 24 hours incubation, the relative fluorescent  
22 intensity of ROS in A549 cells treated with  $1 \mu\text{g ml}^{-1}$  of cisplatin is 7.21. In contrast, the  
23 nanoneedle-treated cells had ROS levels of 14.03 and 15.18 at 12 and 24 hours, respectively,  
24 after treatment. When cells were treated by nanoneedles and cisplatin, the ROS levels of  
25 A549 cells were elevated to 16.72 and 23.77 at 12 and 24 hours, respectively, after treatment.

1 From the fluorescence images shown in Figure 6b, we can see that, after 12 or 24 hours  
2 incubation, nanoneedle array treatment could obviously elevate intracellular ROS production.

### 3 **2.7. Nanoneedle treatment induces increased intracellular G6PD level**

4 The results in Figures 4 and 6 show that nanoneedle treatment leads to slightly increased  
5 apoptosis and higher ROS contents. When cells are jointly treated by nanoneedles and drug  
6 molecules (e.g., cisplatin), these findings may partially explain the reason of the improved  
7 drug efficacy when aided with nanoneedle treatment. However, according to our previous  
8 cytotoxicity measurement, the nanoneedle treatment alone will not lead to measurable cell  
9 death with MTT assay. We speculate that the elevated ROS level and apoptosis signal could  
10 trigger the redox components to counteract the negative effect of nanoneedle treatment to the  
11 physiology of the cells. The intracellular redox potential is determined by the ratio of oxidants  
12 and reductants. Nicotinamide adenine dinucleotide phosphate (NADPH) is the principal  
13 intracellular reductant that can be used by many enzymes for reducing power in cells such as  
14 glutathione reductase and catalase.<sup>[28]</sup> G6PD is the first and rate-limiting enzyme of pentose  
15 phosphate (PP) signal pathway to determine the NADPH production by controlling the  
16 metabolism of glucose via PP signal pathway.<sup>[29]</sup> G6PD and NADPH have an essential role to  
17 counterbalance the oxidative stress and protect cells from oxidative stress-induced  
18 apoptosis.<sup>[30-32]</sup> To monitor the intracellular G6PD levels, A549 cells were treated with a  
19 diamond nanoneedle array and the measurement was performed at 3, 6, 12 and 24 hours after  
20 the treatment. For untreated cells, the test was also carried out at all different time points for  
21 comparison. As shown in Figure 7, compared with the control groups, the intracellular G6PD  
22 level in A549 cells are dramatically increased with time increasing and reaches the highest  
23 level at 12 hours and slowly drops to normal level after 48 hours culture. (Figure S5,  
24 Supporting Information). However, combining with the result shown in Figure 4, we  
25 speculate that the increased G6PD activity still could not completely balance the high ROS  
26 content, and therefore the treatment still led to slight elevation of apoptosis signal pathway.

## 1 **2.8. Depolarization of mitochondrial membrane potential**

2 After knowing that the diamond nanoneedle array treatment can cause the production of ROS,  
3 we next studied the possible reasons. Mitochondria is an important organelle that plays a  
4 central role in cellular viability, such as heat generation, cell growth, cell death via apoptosis,  
5 passive proton leak, formation and degradation of ROS.<sup>[33-37]</sup> Via different experimental  
6 models, it has been repeatedly demonstrated that a lower mitochondrial membrane potential is  
7 observed with a simultaneously increase of ROS production.<sup>[38, 39]</sup> In this study, we used the  
8 MitoProbe JC-1 assay kit to test mitochondrial membrane potential. JC-1 is a lipophilic dye  
9 and can selectively enter into mitochondria with a reversible change of color from green (529  
10 nm) to red (590 nm) as the membrane potential increases. The mitochondrial membrane  
11 potential was detected at three different time points (0, 12 and 24 h). At first, the ratio of  
12 red/green (R3/R2) decreases immediately after the nanoneedle arrays treatment. But after 12  
13 hours, or longer (24 hours), the mitochondrial membrane potential recovers to normal ratio  
14 when compared with control group (**Figure 8**). This indicates that the depolarization of  
15 mitochondrial membrane potential could be one of the reasons of the generation of ROS.  
16 However, some production of non-mitochondrial ROS could not be completely excluded,  
17 such as the membrane-bound NADPH oxidase<sup>[40]</sup>. Further experiments will be necessary to  
18 understand the generation of ROS and its influence on the cell physiology.

19

## 20 **3. Conclusion**

21 In summary, our results show that centrifugation force regulated diamond nanoneedle  
22 treatment of cell suspensions could deliver molecules into cells in an efficient way.  
23 Attractively, the approach does not induce noticeable cytotoxicity indicated by the viability of  
24 nanoneedle array treated cells and genotoxicity suggested by the lack of pH2AX expression.  
25 However, the treatment leads to increased ROS content and cell apoptosis. The elevated  
26 Annexin V binding to the cell membranes, shown by the increased apoptosis of cells, reveals

1 that diamond nanoneedle array disruption can induce lipid scrambling and then affect the  
2 intracellular metabolic signal pathways. For the cells only treated by our nanoneedle arrays,  
3 these do not induce much cytotoxicity. The reason might be that their damaging to the cells is  
4 partially counteracted by the elevation of G6PD. It is possible that the production of ROS  
5 content during nanoneedle array treatment is partially caused by the decreased mitochondrial  
6 membrane potential (**Figure 9**). Importantly, by using calcein-AM and PI double staining, we  
7 explored that the cell membranes should fully recover within 40 minutes after diamond  
8 nanoneedle treatment.

9 In summary, this is the first report in which we systematically investigated the influence  
10 of diamond nanoneedle array treatment on the metabolic signal pathways of cells. It could  
11 provide a new arena for exploiting the nanoneedle application.

12

#### 13 **4. Experimental**

14 *Fabrication of diamond nanoneedle arrays:* Diamond nanoneedle arrays were produced  
15 by a top-down method. Firstly, polycrystalline diamond thin film was fabricated by  
16 Microwave Plasma Chemical Vapor Deposition (MPCVD). N-type silicon wafer (100) –  
17 oriented, 1-10  $\Omega$ , was polished with diamond particle paste (0.25  $\mu\text{m}$ ) was used as a substrate.  
18 Silicon substrate was then put into ultrasonic bath with alcohol and acetone for cleaning.

19 The ASTeX MWCVD reactor connected with a 1.5 kW microwave generator was  
20 accounted for deposition of polycrystalline diamond film. Methane ( $\text{CH}_4$ ) and hydrogen ( $\text{H}_2$ ),  
21 in the ratio 1% and 99% were being used as gas precursor, with the total flow rate of 300  
22 SCCM. The temperature, pressure and microwave power were maintained at 800  $^\circ\text{C}$ , 30 Torr  
23 and 1200 W, respectively, for 24 hours. Secondly, after synthesis of diamond film, it was  
24 etched to diamond nanocone arrays by using Electron cyclotron resonance Microwave Plasma  
25 Chemical Vapour Deposition (ECR-MPCVD). Around 875G B-field were generated by an

1 external magnetic coil to the centre of the chamber. Gases mixture of 70% He, 25% Ar, 5%  
2 N<sub>2</sub> were used to induce plasma by 1400 W microwave source. The temperature and pressure  
3 were kept at 400 °C and  $2 \times 10^{-3}$  Torr, respectively.

4 *Cell cultures:* A549 cells (ATCC) were maintained in Dulbecco's modified Eagle  
5 medium (DMEM, Life Technology) supplemented with 10% fetal bovine serum Hyclone, L-  
6 glutamine and 1% penicillin/streptomycin at 37 °C with 5% CO<sub>2</sub>. Doxorubicin resistant  
7 MCF7 cell line (DR-MCF7) was cultured in full medium with the addition of 2 µg ml<sup>-1</sup>  
8 doxorubicin (Sigma) for achieving doxorubicin resistance.

9 *Diamond nanoneedle arrays to facilitate intracellular delivery:* Adherent cells were  
10 firstly trypsinized to obtain a cell suspension. Then the cell suspension was placed onto a  
11 diamond nanoneedle array located in a 24-well cell culture plate ( $2 \times 10^4$  cells per well)  
12 followed by centrifugation at 120 g for 5 min at room temperature (CT15RT Eppendorf,  
13 German). This is the standard nanoneedle treatment condition in this paper unless otherwise  
14 specified. The experiments were performed in 3 groups for each cargo material of cisplatin  
15 and dextran-FITC. The first and second group contained cargo molecules of cisplatin (or  
16 dextran-FITC), but only one group of cells experienced diamond nanoneedle treatment. The  
17 third group did not have any cargo molecules and acts as an untreated negative control. In this  
18 paper, untreated control group means that the cells were not treated with centrifugation. After  
19 centrifugation, the cell suspension was removed from the diamond nanoneedle arrays and then  
20 harvested for subsequent operations.

21 *Cytotoxicity assay:* The releasing level of glucose 6-phosphate (G6PD) was measured by  
22 the G6PD release assay kit (Invitrogen, USA). Briefly, after nanoneedle treatment, cells were  
23 transferred to a 96-well plate and cultured for 6 hours in a volume of 50 µl. Then 50 µl of  
24 resazurin was added into the plate to a concentration 15 µM. After 10-30 minutes incubation  
25 at 37 °C, the fluorescence intensity was read by a fluorescence microplate reader (BMG



1 Polarstar Optima, Ortenberg, Germany) with appropriate excitation (530-560 nm) and  
2 emission filters (580-600 nm).

3 Cell viability was tested by MTT assay. After nanoneedle treatment, cells were  
4 transferred into a 96-well plate and cultured for 48 hours at 37 °C. Then the medium was  
5 removed and replaced with 100 µl of fresh culture medium containing no cargo molecules. 10  
6 µl of 12 mM MTT stock solution was added into each well followed by incubation at 37 °C  
7 for 4 hours. Subsequently, all media were removed and replaced with 100 µl DMSO. Finally,  
8 the absorbance at 540 nm of each sample was measured by a UV-Vis-IR microplate reader  
9 (Powerwave XS MQX200R, Leicestershire, England).

10 *Flow cytometry:* The apoptosis assay was conducted according the protocol of Alexa  
11 Fluor 488 annexin V/dead cell apoptosis kit (Invitrogen, Carlsbad, USA). A 488 nm laser  
12 served as an exciting source for annexin V-FITC (FL1, 530/30 nm) and PI (FL2, 575/24 nm)  
13 (BD FACSCalibur, flow cytometer).

14 For studying the delivery of doxorubicin into MCF/ADR cells, three groups were  
15 conducted. The first group of MCF7/ADR cells was incubated with 2 µg ml<sup>-1</sup> doxorubicin for  
16 5 minutes without diamond nanoneedle treatment. The second group was treated by diamond  
17 nanoneedle arrays for 5 minutes with the addition of 2 µg ml<sup>-1</sup> doxorubicin. Untreated  
18 MCF7/ADR cells were used as control. Then three groups of cell suspension were collected  
19 and washed by PBS for two times to remove the extra doxorubicin and re-suspended in PBS  
20 for flow cytometry analysis with excitation at 488 nm and emission filter at 580 nm.

21 ROS was measured by the CellROX Green Reagent (Invitrogen, Carlsbad, USA) in  
22 living cells. CellROX green reagent was added into a medium to a final concentration of 5  
23 µM and incubated with cells for 30 minutes at 37 °C. Then cells were washed for three times  
24 with PBS for flow cytometry studying (EX/EM 488/520 nm).

25 *Fluorescent microscopy:* After centrifugation, cells delivered with dextran fluorescent  
26 488 (10,000 MW, Invitrogen, Carlsbad USA) were incubated with 2 µl of 20 mM

1 Hoechst33342 (Invitrogen, Carlsbad, USA) for 20 minutes for nuclei staining at 37 °C.  
2 Cellular fluorescence was monitored using fluorescence microscopy.

3 *pH2AX detection and Image-iT Dead Green staining:* The level of pH2AX was measured  
4 by HCS DNA damage kit (Invitrogen, Carlsbad, USA) with the following procedures. First,  
5 after being treated by a diamond nanoneedle array, cells were transferred to a 96-well plate  
6 and cultured for 3, 6 or 24 hours. Second, the cells were stained with image-iT Dead Green  
7 viability stain working solution for 30 minutes at 37 °C. Third, the cells were fixed with 4%  
8 paraformaldehyde solution for 15 minutes, washed with PBS and incubated with a  
9 permeabilization solution of 15 µl of Triton X-100 to 6 mL PBS at room temperature. Fourth,  
10 the samples were washed with PBS and incubated with blocking buffer containing 1% BSA  
11 for 60 minutes. Fifth, the samples were incubated with the primary pH2AX antibody for 60  
12 minutes and washed with PBS for three times. Sixth, the secondary antibody Alexa Fluor 555  
13 goat anti-mouse IgG (H+L) was added and incubated for another 60 minutes. Seventh, the  
14 secondary antibody was removed and the cells were rinsed with PBS. Eighth, 100 µl of fresh  
15 PBS was added into each well for analysis. The samples were scanned by microplate reader  
16 (BMG Polarstar Optima, Ortenberg, Germany) with filters appropriate for Hoechst (350/461  
17 nm), Image-iT Dead Green viability stain (488/515 nm) and Alexa Fluor 555 goat anti-mouse  
18 IgG (555/565 nm).

19 *Calcein-AM and PI staining:* After being treated by the diamond nanoneedle arrays,  
20 A549 cells were suspended in PBS. Calcein-AM and PI were added into the cell suspensions  
21 at different time points and incubated at 37 °C for 20 minutes. Then the cells were washed by  
22 PBS for two times and the cellular fluorescence was monitored by fluorescence microscopy  
23 and analysed with ImageJ. A549 cells without nanoneedle treatment were set as a control  
24 group.

25 *Statistical Analysis:* Experiments were repeated at least three times. For each set of the  
26 experiments, results were expressed as mean values  $\pm$  standard deviation. Statistical

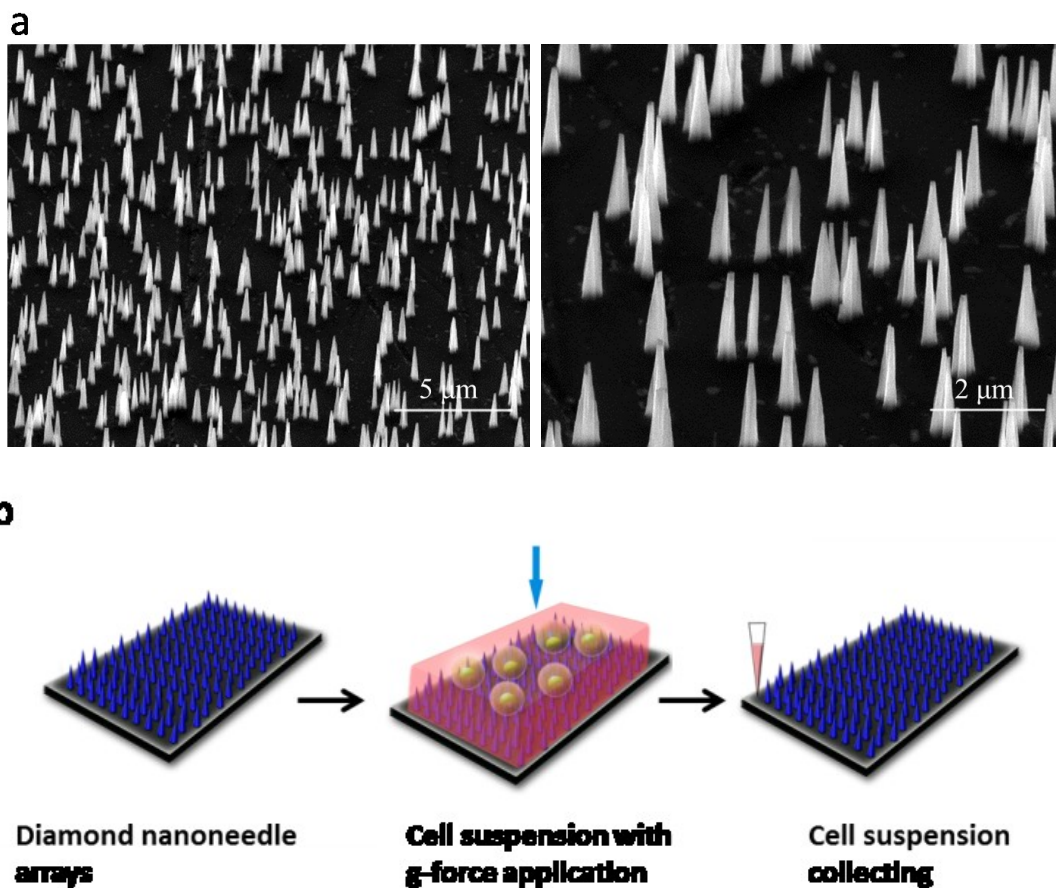
1 significance was calculated by either the student t-test for paired comparisons or the one-way  
2 ANOVA for multiple comparisons. A  $p$ -value  $< 0.05$  was considered as significance.

3  
4 Received: ((will be filled in by the editorial staff))  
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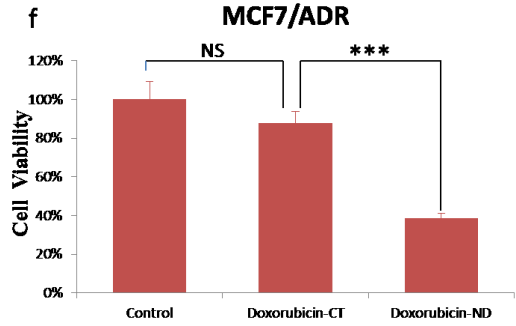
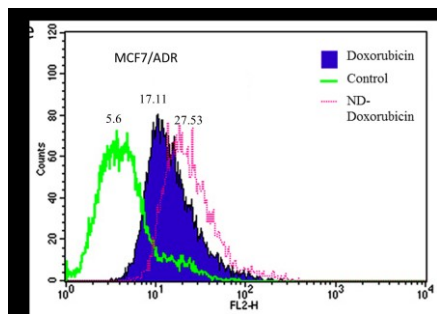
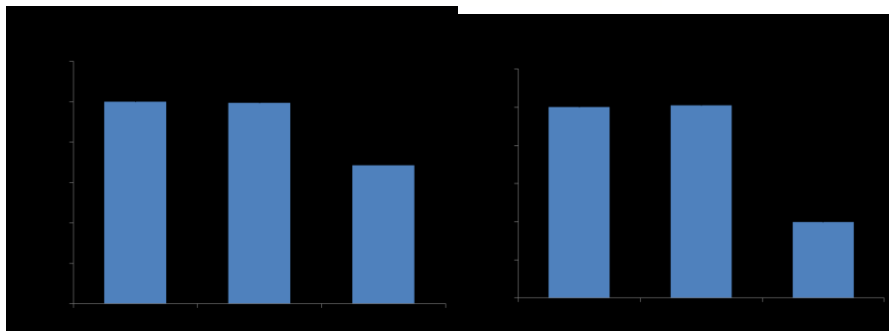
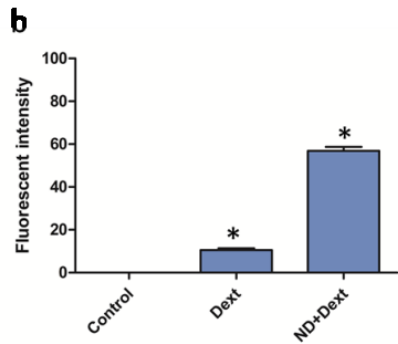
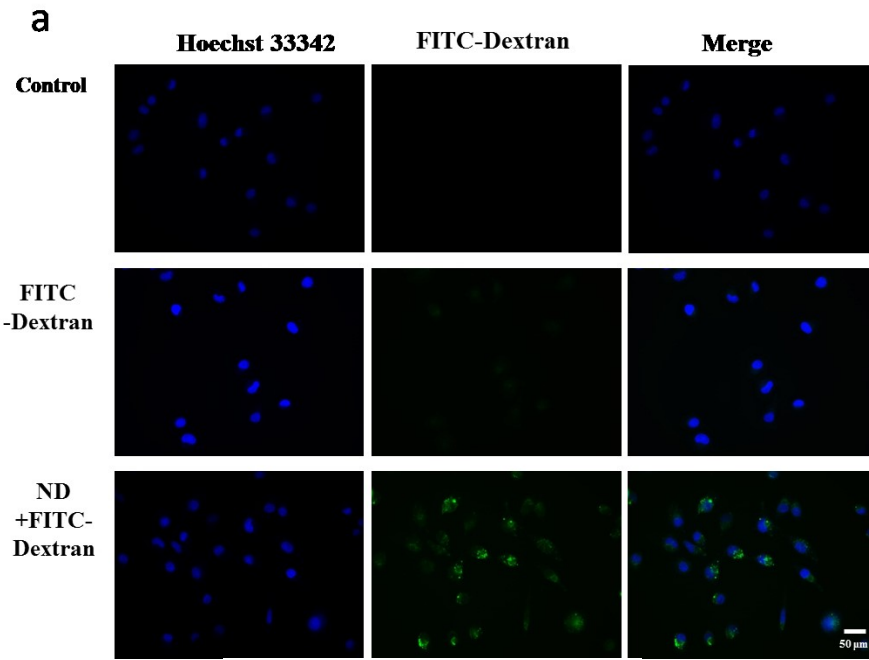
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- 16



3 **Figure 1.** (a) SEM images of diamond nanoneedle arrays. The scale bars indicate 5 (left) and  
4 2  $\mu\text{m}$  (right). (b) Schematic representation of diamond nanoneedle mediated intracellular  
5 delivery with centrifugal g-force application. A cell suspension is applied to the surface of a  
6 diamond nanoneedle array and then a centrifugation force is used to control the disruption of  
7 the cell membranes for facilitated intracellular delivery. At the end of the process, the cell  
8 suspension is collected.

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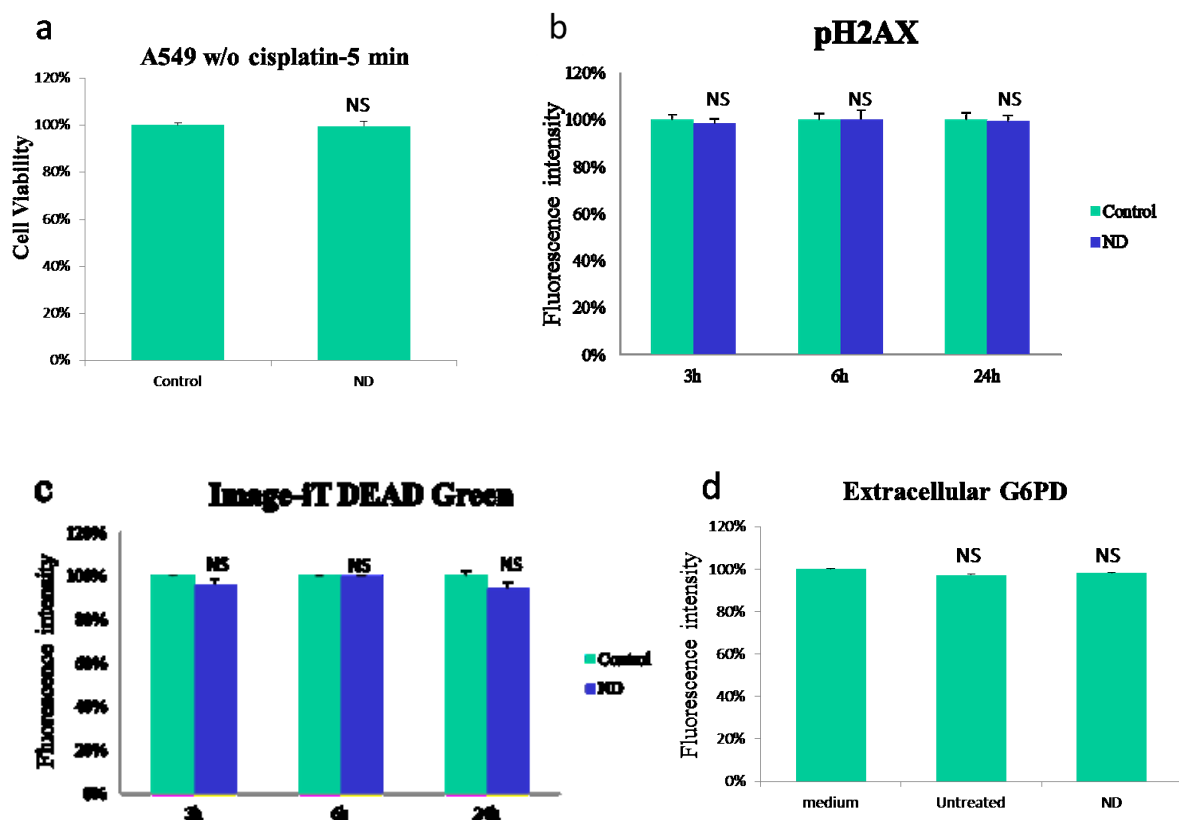
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8 **Figure 2.** Cytosolic delivery by diamond nanoneedle arrays. (a) Fluorescence images of A549  
 9 cells after delivery of FITC-Dextran (10,000 MW) under different conditions (no treatment,

1 incubation with FITC-Dextran, incubation with FITC-Dextran and diamond nanoneedle  
2 treatment). Hoechst 33342 was used to stain the nucleic acid. Green signal indicates the  
3 fluorescence of FITC-Dextran. (b) Quantification of fluorescent intensity within the cells by  
4 using Image-J. (c) and (d) Cell viability of A549 cells under different conditions (no treatment,  
5 incubation with  $0.2 \mu\text{g ml}^{-1}$  cisplatin (cisplatin-CT), treated with diamond nanoneedle arrays  
6 (ND) and incubation with  $0.2 \mu\text{g ml}^{-1}$  cisplatin. MTT assays were conducted at 48 hours after  
7 incubation. (c) nanoneedle arrays treatment of cells for 30 seconds. (d) nanoneedle arrays  
8 treatment of cells for 5 minutes. ( $*p < 0.01$ ) (e) The delivery of doxorubicin to MCF7/ADR  
9 cells with and without nanoneedle treatment. Flow cytometry analysis of the fluorescence  
10 intensities of doxorubicin were tested immediately after drug delivery. (f) Cell viabilities of  
11 MCF7/ADR cells treated with  $1 \mu\text{g ml}^{-1}$  doxorubicin with the aid of diamond nanoneedles  
12 (Doxorubicin-ND) or without using nanoneedles (Doxorubicin-CT). Untreated MCF7/ADR  
13 cells were used as negative control. ( $*p < 0.01$ ; NS: non-significant)  
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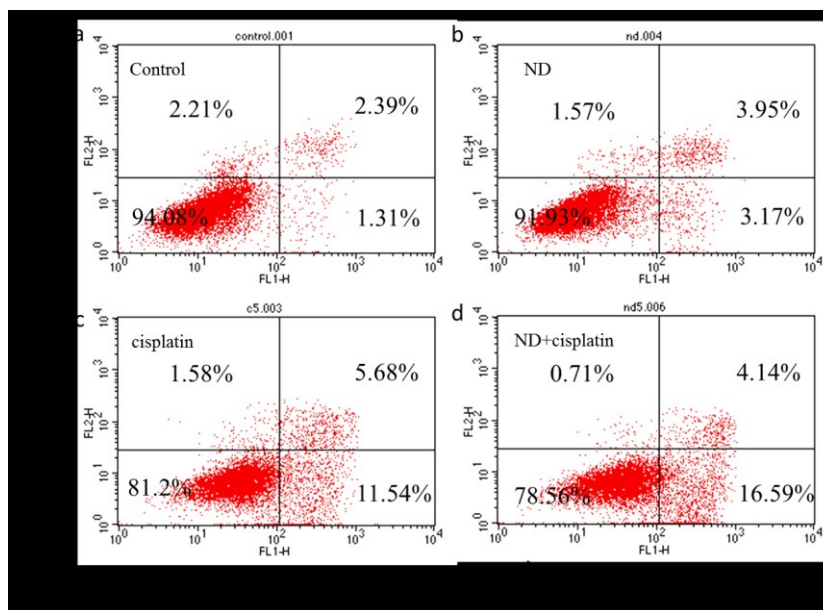




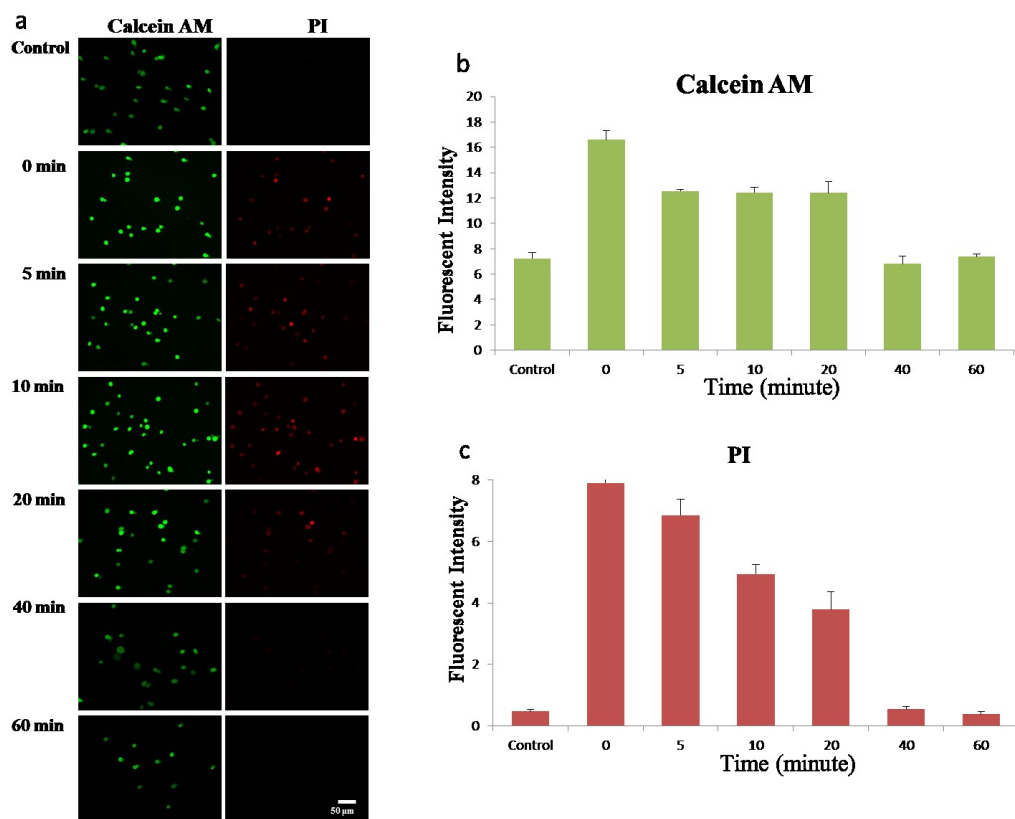
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3 **Figure 3.** (a) Viability of A549 cells treated with diamond nanoneedle arrays. Cell viability  
 4 was detected by MTT assay. Untreated cells were used as a control group. (b) Genotoxicity  
 5 assay and (c) cell membrane integrity analysis. After being treated by diamond nanoneedle  
 6 arrays, A549 cells were cultured in 96-well plate for 3, 6 or 24 hours. DNA double-strand  
 7 breaks were investigated by monoantibody pH2AX detection. The fluorescence intensity of  
 8 pH2AX signal was detected by using a TRITC filter (EX/EM 555/565 nm). The integrity of  
 9 plasma membrane was evaluated by image-iT DEAD Green viability staining. The  
 10 fluorescence intensity was assessed by a FITC filter (EX/EM 488/515 nm). (d) Detection of  
 11 Extracellular G6PD level in A549 cells treated with a nanoneedle array. A549 cells were  
 12 treated with a nanoneedle array (ND) followed by culturing of 24 hours, and then the  
 13 extracellular G6PD release was measured (EX/EM 530/590 nm). Cell culture medium alone  
 14 and untreated cells were used as control groups. (\* $p < 0.01$ ; NS: non-significant)

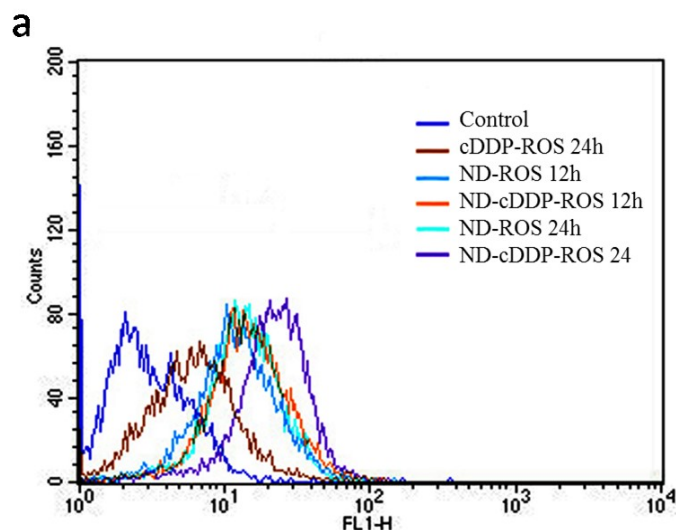


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 2 **Figure 4.** Flow cytometry analysis of apoptosis and necrosis of A549 cells. (a) Untreated cells;  
 3 (b) Cells treated with a diamond nanoneedle array alone; (c) Cells incubated with  $5 \mu\text{g ml}^{-1}$  of  
 4 cisplatin for 24 hours. (d) Cisplatin was added to a cell suspension with a concentration of  $5$   
 5  $\mu\text{g ml}^{-1}$  and the suspension was applied to a diamond nanoneedle array for treatment for 5  
 6 minutes. Subsequent, the cell suspension was collected and cultured in a 96-well plate for 24  
 7 hours. The analysis was conducted after 24 hours incubation. For all groups, A549 cells were  
 8 stained and analysed by 488 nm excitation with 530/30 nm and 575/24 nm bandpass filters  
 9 and collected by means of a standard  $100 \mu\text{l minute}^{-1}$  collection rate. Early apoptotic cells are  
 10 shown in the lower right quadrant, and necrotic cells are shown in the upper left quadrant.  
 11

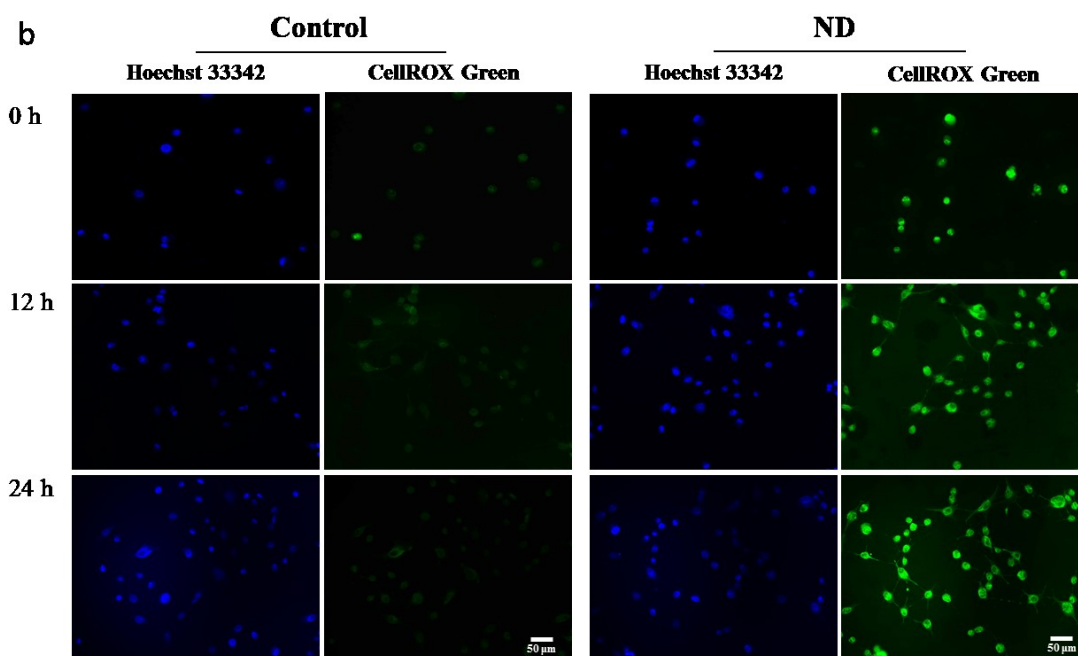


1  
 2 **Figure 5.** Cytosolic delivery of calcein-AM and PI in A549 cells. (a) Calcein-AM (left) and  
 3 PI (right) fluorescent images of living A549 cells when the chemicals were added at different  
 4 time points after the cells being treated with nanoneedles. (b) Quantification of calcein-AM  
 5 fluorescent intensity of different groups. (c) Quantification of PI fluorescent intensity at  
 6 different time points. Untreated A549 cells were set as a control group.

7



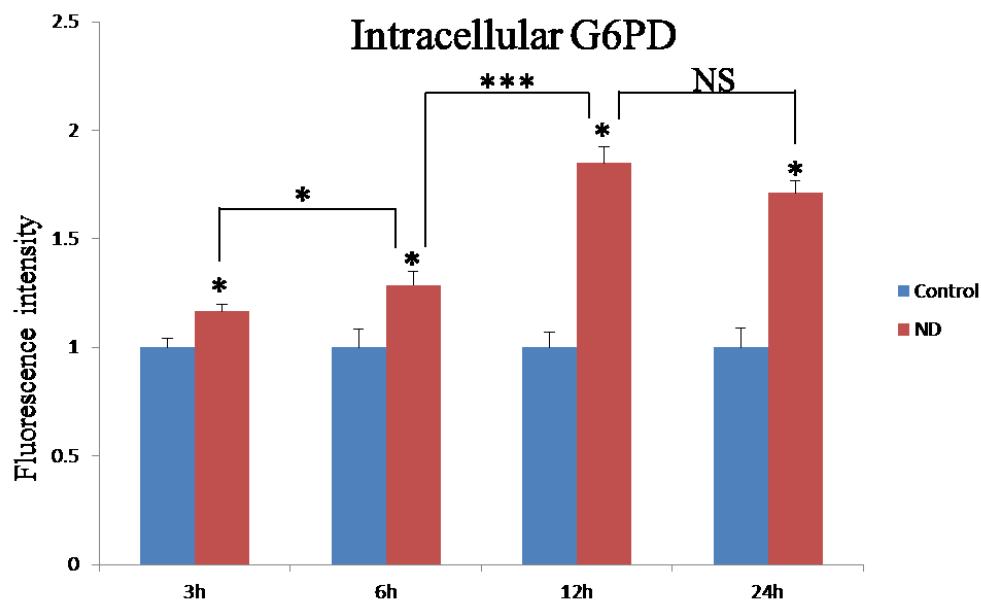
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2

3 **Figure 6.** ROS content detection in A549 cells with nanoneedle array treatment. (a) After  
 4 incubating for 12 or 24 hours, flow cytometry analysis was conducted to evaluate the ROS  
 5 content in A549 cells. Cells treated with or without  $1 \mu\text{g ml}^{-1}$  cisplatin (cDDP) addition and a  
 6 nanoneedle array (ND) as indicated in the figure were stained with  $5 \mu\text{M}$  CellROX Green  
 7 reagent (EX/EM 485/520 nm). (b) Fluorescence images of ROS in A549 cells with or  
 8 without nanoneedle array treatment (ND). Hoechst 33342 was used to stain cell nucleus. The  
 9 cells without nanoneedle array treatment were used as control group. 0 h means immediately  
 10 after nanoneedle treatment.

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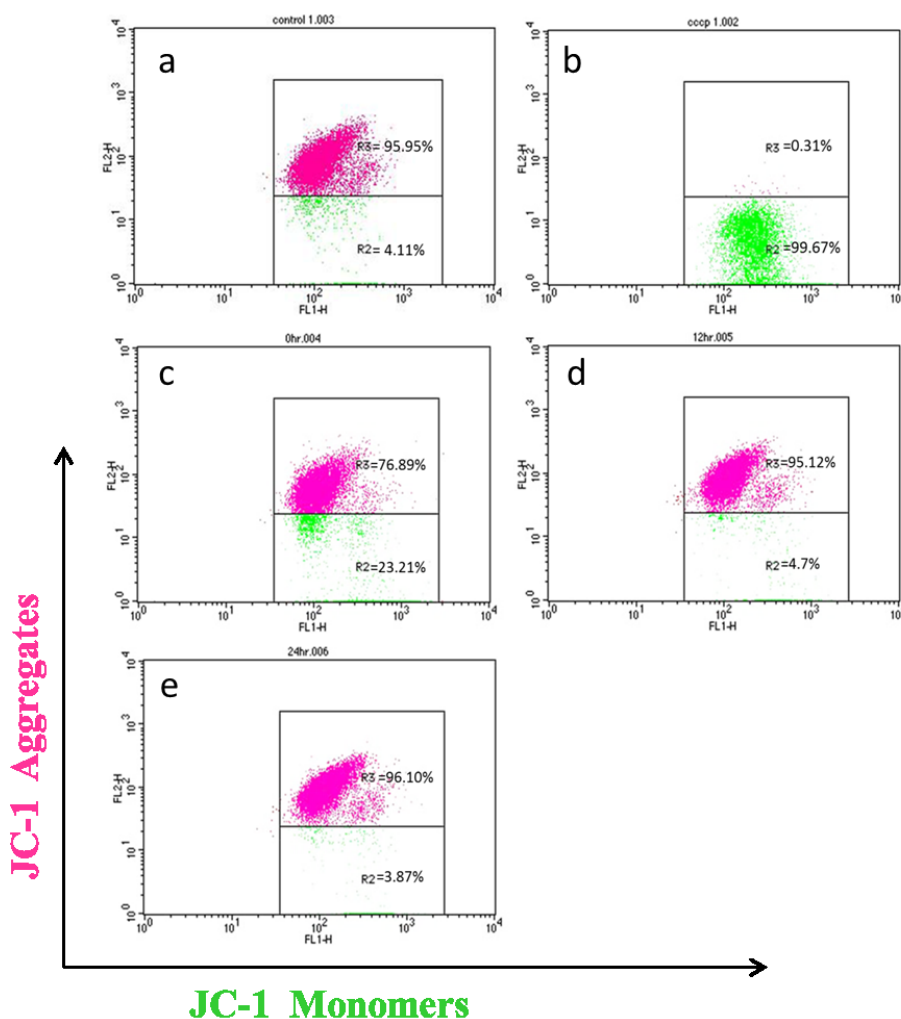
3 **Figure 7.** Intracellular G6PD activity assay. Untreated A549 cells were used as control group.

4 After being treated by a nanoneedle array platform (ND), A549 cells were cultured in 96-well

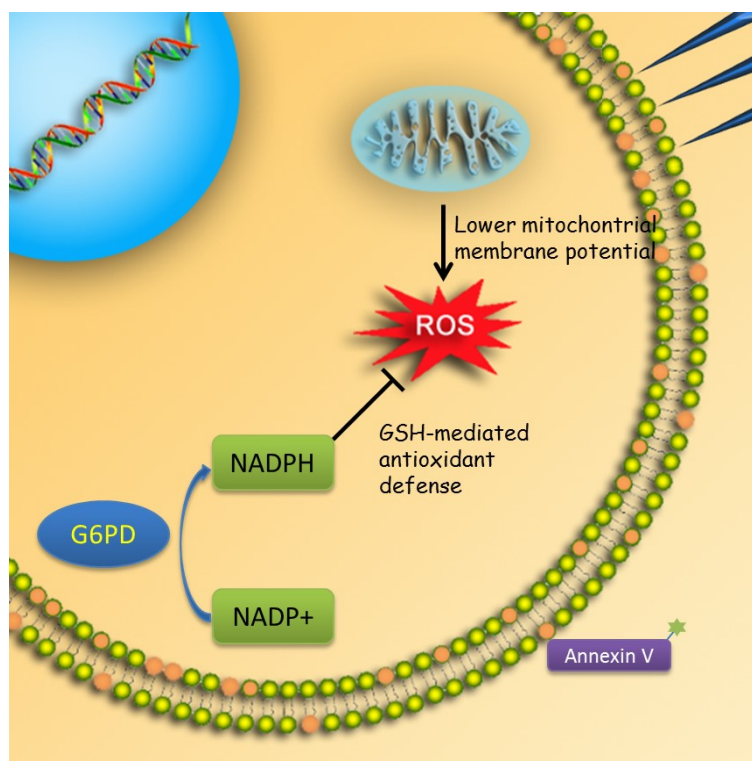
5 plate for 3, 6, 12, and 24 hours as indicated. Then the cells were lysed and the activity of

6 G6PD was measured in a microplate reader (EX/EM 530/590 nm). (\* $P < 0.01$ ; NS: non-

7 significant)



1  
 2 **Figure 8.** Flow cytometry assay of mitochondrial membrane potential. (a) Untreated A549  
 3 cells were used as positive control. (b) Untreated A549 cells with 50  $\mu$ M cccp (carbonyl  
 4 cyanide 3-chlorophenylhydrazone) were used as negative control. Following the nanoneedle  
 5 array treatment, A549 cells were stained with 2  $\mu$ M JC-1 immediately (c), or after 12 (d) or 24  
 6 hours (e) incubation. Cells were analysed on flow cytometer using 488 nm excitation with  
 7 530/30 nm and 585/42 nm band-pass emission filters. The intensity of green and red  
 8 fluorescence intensities are shown as R2 and R3, respectively.



1  
2 **Figure 9.** Schematic representation of the influence of diamond nanoneedle array treatment  
3 on the intracellular metabolic system. Following the diamond nanoneedle arrays penetration,  
4 the phosphatidylserine is exposed to the extracellular environment and can bind with Annexin  
5 V. The nanoneedle treatment leads to a significant decrease of mitochondrial membrane  
6 potential. As a result, a high content of ROS is produced under the increased oxidant stress.  
7 Under this oxidant environment, the activity of intracellular redox enzymes G6PD is aroused  
8 to counterbalance the high production of ROS. In the process, NADPH is synthesised and  
9 utilized by the glutathione (GSH) mediated antioxidant defence.