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TiO_2 nanotubes: photocatalyst for cancer cell killing

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The present work reports on the use of self-organized TiO_2 nanotube layers for the photocatalytic killing of cancer cells. TiO_2 nanotube layers with different dimensions (diameter 50 nm and 100 nm, thickness 800 nm and 1.3 µm, respectively) were grown by anodization of Ti. Upon low dose of UV irradiation, the vitality of cancer cells cultured on these nanotube layers was significantly affected – the cells reduced their shape and size and a significant amount of the dead cells was found. These results demonstrate that self-organized TiO_2 nanotube layers can be used for photo-induced cancer cell killing.

The use of TiO_2 as a photocatalyst that is able to destroy unwanted and harmful organic compounds in contaminated air or water has been extensively studied since the 1972 report of Fujishima and Honda on the photo-induced and electrochemically assisted decomposition of water on TiO_2 surfaces [1]. Throughout the past three decades, many different groups have investigated the strong oxidizing ability of TiO_2 that is based on the high energy of the photo-induced electron (e^-)–hole (h^+) pairs upon UV light excitation [2–4]. Apart of unwanted recombination, the charge carriers may form reactive oxygen species (ROS) including O_2^- and in particular $OH^\bullet$ radicals of a highly oxidative nature.

In 1985, Matsunaga et al. showed for the first time the ability of UV-irradiated platinized TiO_2 nanopowder to kill bacterial cells in an aqueous environment [5]. They explained the cell death by limited or aborted respiratory functions as a result of membrane damage by the products of the photo-induced reactions on the TiO_2 surface. Since then, different research groups have investigated the photo-induced devitalization of various cells with TiO_2 nanoparticles and provided different additional explanations for the mechanism of cell death [6–10].

In 1999, an approach for synthesis of highly ordered, vertically oriented TiO_2 nanotubes on Ti and Ti-alloy substrates has been reported [11]. It is based on anodization of the substrates in a fluoride containing electrolyte under conditions that lead to self-organization. Up to now, several generations of nanotubes have been brought forward [12–14]. In recent reports we have shown that these self-organized TiO_2 nanotubes exhibit very high photocatalytic efficiency that can be used for the decomposition of organic compounds in the contact mode (on the Ti substrate) [15] or in flow-through UV-irradiated membranes [16]. The decomposition efficiency can be further enhanced by using a bias voltage [17], or by coating the nanotubes with noble metal nanoparticles [18]. Most recently we showed that living cells adhere, spread and grow on the surface of nanotubes [19].

In the present work, we study the feasibility to use the nanotube surface for enhanced photo-induced cancer cell killing. For this purpose we formed nanotube layers with two different average tube diameters (50 nm and 100 nm), on which we plated HeLa G cancer cells. After incubating the cells and UV exposure we measured the changes in the cell morphology and viability.

Figure 1 shows SEM top-view images of TiO_2 layers used in this work. The layers were grown by anodization in 0.27 M NH_4F/glycerol/water electrolytes at 10 V (Fig. 1a) and 20 V (Fig. 1b) using a previously reported approach

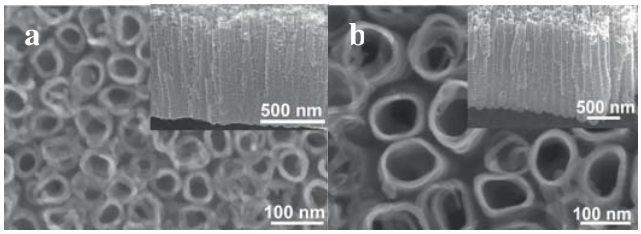

Figure 1 SEM top view images of TiO_2 nanotube layers used in this work. The insets show cross-sectional views. The average tube diameters and average layer thicknesses are (a) 50 nm and 800 nm, (b) 100 nm and 1.3 μm, respectively.

[20]. Prior to the photocatalytic experiment shown schematically in Fig. 2, the human cervical carcinoma HeLa G cell line was grown in DMEM (4.5 g/l glucose) (PAA) supplemented with 10% heat-inactivated fetal bovine serum (Biowest), penicilin (20 U/ml) and streptomycin (20 μg/ml). The cells were cultured at 37 °C in a 5% CO_2 humidified atmosphere. Ti foils with TiO_2 nanotube layers on their surface were inserted into wells of a 6-well tissue culture plate (TPP), sterilized in 70% ethanol for 30 min, then washed with PBS and air-dried. HeLa G cells were plated on TiO_2 nanotubes at the density of 1×10^6 cells per well (in 2 ml of culture medium). After 24 h 1.4 ml of medium was aspirated and center of the sample was irradiated with UV light (microscope Nikon E400, with BP filter 350 ± 25 nm, without objective) for 5 min. The distance between the light source and the samples was approx. 7 cm. The intensity of the light was 5 mW/cm^2 measured by Field Max II (Coherent, Portland, USA). Subsequently, 1.4 ml of culture medium was added and cells were incubated for additional 5 h in cell culture incubator (recovery period).

Afterwards, cells were fixed with 4% paraformaldehyde solution for 10 min, stained with phalloidin conjugated with Alexa-488 (Molecular Probes) (1:100) and DAPI (Sigma) (1:1000). Immunological staining was performed according to a previously published protocol [21]. The staining was visualized using the Nikon E-400 epifluorescence microscope (objective Plan 10×/0.25); digital images were acquired with a CCD-DS U1 camera. Representative images are shown in Fig. 3. Cells cultured on both types of TiO_2 nanotube layers without UV treatment revealed a similar morphology; however, they were slightly smaller than cells on the glass control. A strong difference was observed after the UV treatment. In this case, cells on TiO_2 nanotube layers showed significant size

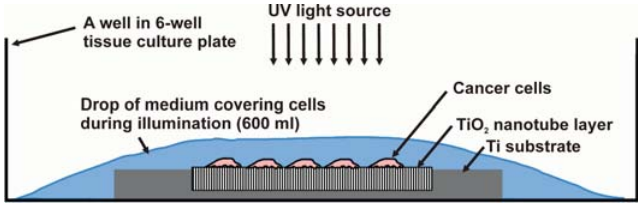

Figure 2 (online colour at: www.pss-rapid.com) Scheme of the setup used during the experiments.

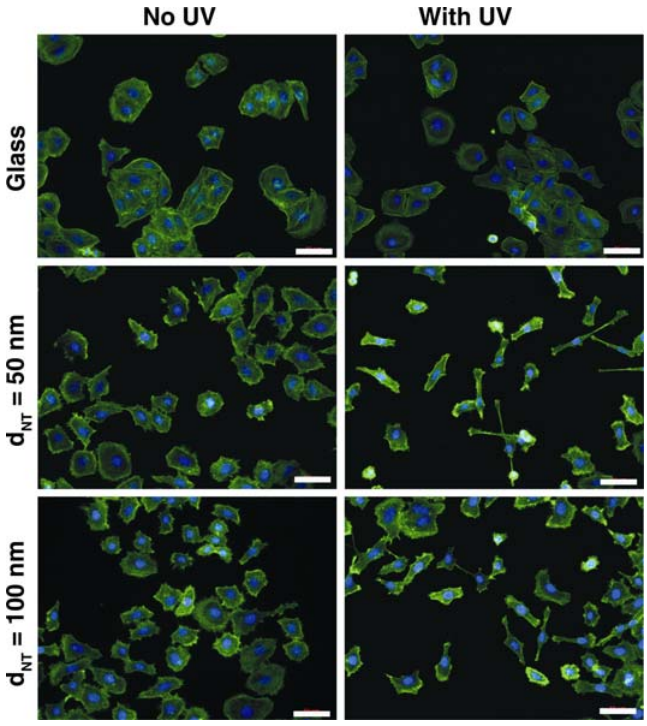

Figure 3 (online colour at: www.pss-rapid.com) Fluorescence microscopy images of the morphology of HeLa G cells cultured on TiO_2 nanotube layers (diameter of nanotubes D_{NT} = 50 nm and 100 nm) and glass with and without UV irradiation. Actin filaments are stained green and nuclei blue. The scale bars are 50 μm.

reduction – some of them turned into rounded shapes, whereas other formed elonged protrusions. For comparison, cancer cells cultured on the glass control, but treated with UV did not exhibit dramatic changes of their morphology and remained comparable in shape and form to the UV-untreated cells on glass slides. Elongated protrusions and cell shrinkage were a result of the photocatalytic activity of the nanotube layers.

A statistical analysis of the images from three independent experiments performed in duplicate was carried out. The total area of an image covered with green signal (fluorescently stained actin) was divided by number of nuclei (blue dots), thus the average cell size was obtained. The results are presented in Fig. 4 as a mean value and standard deviation (SD). It can be seen that HeLa G cells cultured on the TiO_2 nanotube layers (both diameters) after photocatalysis are smaller than untreated cells cultured on the same surface. However, in this case the differences between the UV-treated and non-treated samples appear not as large as the changes in shape shown in Fig. 3.

Images of cells as well as quantitative image analysis data show that the TiO_2 nanotube layers themselves have only a minor effect on cell morphology (cell size), in agreement with previous data by Park et al. [19]. However, in Fig. 4 we found that the average cell area is only slightly larger on the 50 nm tubes than on the 100 nm tubes; i.e. a less pronounced effect than in the Park study [19]. This

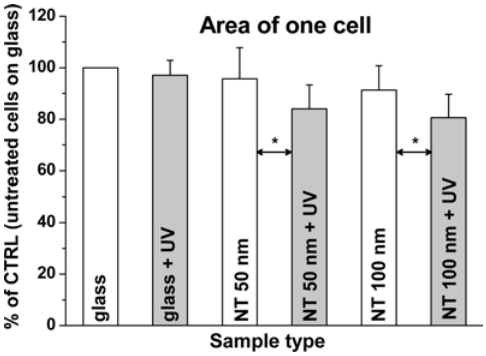

Figure 4 Average cell areas of HeLa G cells cultured on TiO_2 nanotube layers (NT, diameter 50 nm and 100 nm) and glass with and without UV irradiation. Image segmentation and analysis was performed using NIS elements 2.30 SP3. Batch processing was done with a custom C^{++} script. In individual images (10 images for each sample) the total actin stained area was divided by number of nuclei on a picture, thus the area of single cell was obtained. Calculated cell areas were related to the control sample (non-treated cells cultured on a glass, taken as 100%). Statistical analysis of the data was performed by a one-way analysis of variance (ANOVA). * Significant difference $p < 0.01$.

may be attributed to usage of different cell types (mesenchymal stem cells in [19] and an epithelial tumor cell line in our study).

After 5 min of UV irradiation and subsequent incubation of the cells for 5 hours, we measured the lactate dehydrogenase (LDH) activity in cell culture medium of different substrates. Using a Cytotoxicity Detection Kit (Roche), the LDH release was calculated as the percentage of LDH activity released from the control sample (non-treated cells cultured on glass, taken as 100%). Figure 5 shows that the activity of LDH in medium (reflecting cells with ruptured membrane = dead cells) is comparable with the cell-cultured TiO_2 nanotube layers of 50 nm diameter with or without UV irradiation. In contrast, cells cultured on TiO_2 nanotube layers with diameter of 100 nm exhibit higher level of LDH after UV radiation compared to the non-irradiated sample.

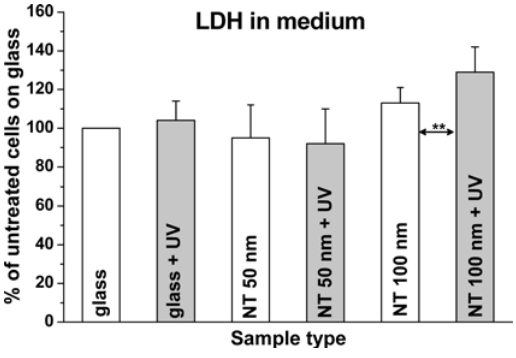

Figure 5 LDH activity measured in medium of HeLa G cells cultured on TiO_2 nanotube layers (diameter 50 nm and 100 nm) and glass with and without UV irradiation. ** Significant difference $p < 0.05$.

This higher amount of LDH is consistent with a higher oxidative stress suffered by the cells plated on TiO_2 nanotubes. Note that in our experimental conditions, the dose and time of exposition are sufficiently low that in the performed control experiments with cells cultured on glass we did not observe any changes after UV exposition.

In conclusion, we demonstrate that TiO_2 nanotube layers can be used as a photocatalyst for killing of cancer cells. The dimensions of the nanotubes play a role for cell adhesion and spreading. A possible application of the nanotubes may be an anticancer treatment, where isolated tubes are administrated to a tumor, followed by a focused UV-light exposition of the tumor. Using UV light as excitation source for the photocatalytic cancer killing requires however a direct access of the light to the TiO_2 nanotubes [22]. However, it has to be pointed out that also X-rays may be used to trigger a photocatalytic reaction on TiO_2 [23], or alternatively C- [24] or N-doped [25] material may be used to obtain effects at longer wavelength (as longer wavelength show a considerably higher penetration depth).

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