Complex cytotoxicity mechanism of bundles formed from self-organised 1-D anodic TiO_2 nanotubes layers

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Abstract

The present study reports on a comprehensive investigation of mechanisms of *in vitro* cytotoxicity of high aspect ratio (HAR) bundles formed from anodic TiO_2 nanotube (TNT) layers. Comparative cytotoxicity studies were performed using two types of HAR TNTs (diameter of ~110 nm), differing in initial thickness of the nanotubular layer (~35 μm for TNTs-1 *vs*. ~10 μm for TNTs-2). Using two types of epithelial cell lines (MDA-MB-231, HEK-293), it was found that nanotoxicity is highly cell-type dependent and plausibly associates with higher membrane fluidity and decreased rigidity of cancer cells enabling penetration of TNTs to the cell membrane towards disruption of membrane integrity and reorganization of cytoskeletal network. Upon penetration, TNTs dysregulated redox homeostasis followed by DNA fragmentation and apoptotic/necrotic cell death. Both TNTs exhibited haemolytic activity and rapidly activated polarization of RAW 264.7 macrophages. Throughout the whole study, TNTs-2 possessing a lower aspect ratio manifested significantly higher cytotoxic effects. Taken together, this is the first report comprehensively investigating the mechanisms underlying the nanotoxicity of bundles formed from self-organised 1-D anodic TNT layers. Except for description of nanotoxicity of industrially-interesting nanomaterials, the delineation of the nanotoxicity paradigm in cancer cells could serve as solid basis for future efforts in rational engineering of TNTs towards selective anticancer nanomedicine.

Keywords: 1-D materials; Biocompatibility; Nanotoxicology; Nanotubes; Titanium dioxide

1. Introduction

Nowadays, society is witnessing unparalleled interest in nanomaterials. This has resulted in a plethora of innovative applications in various spheres of medicine and industry [1-3]. Among these, titanium dioxide (TiO_2) has been successfully used in a number of industrial products, particularly as pigment in food industry and dermatological preparations. It is worth noting that exceptional photocatalytic properties of TiO_2 also triggered enormous interest in the utilisation of TiO_2 for energy conversion and storage, representing essential parts of the concept of sustainable environment [4].

The most investigated form of TiO_2 are undoubtedly TiO_2 0-D materials (nanoparticles) with a wide spectrum of morphologies, surface characteristics and inner structures [5-7]. 1-D structures, having one dimension outside the nanoscale (typically length), *e.g.* nanorods or nanotubes (NTs) represent another promising group of TiO_2-based nanomaterials [8]. In general, 1-D materials exhibit improved reactant/unidirectional charge/ion transport and enhanced mechanical integrity [9]. Due to a large number of advantageous properties [10-12], such as high aspect ratio, scalability and ability to grow in a controlled geometry, 1-D TiO_2 nanotube (TNT) layers are considered among the most promising nanomaterials for utilisation in industrial photocatalysis and biomedicine [13-15].

Advances in nanoengineering have resulted in a dire need of delineating the fundamental biological mechanisms of communication between nanomaterials and biological systems [16]. In general, nanoscaled TiO_2 have been considered as low toxicity material; however, our recent study revealed that small (diameter of ~6 nm) TiO_2 nanoparticles exhibit inherent cytotoxicity for mammalian cells [17] and similar findings have been reported in a number of studies assessing cytotoxicity of TiO_2 nanoparticles in distinct experimental models [18-20]. Importantly, apart of direct cytotoxicity caused by formation of reactive oxygen species (ROS), followed by DNA damage, TiO_2 nanoparticles have been shown to induce epigenomic toxicity

[21] or to disrupt exocytosis by hindering membrane ion exchange [22]. In addition, it has been demonstrated that the cytotoxicity positively correlates with size (surface area) of TiO_2 nanoparticles [23-25] and is also substantially affected by their crystalline phase [17, 26] and surface chemistry/doping [27].

Majority of studies focused on TNT layers investigate their ability to bestow antimicrobial activity for Ti-based orthopaedic implants [28, 29]. Similarly to TiO_2 nanoparticles, to date, reports on the cytotoxicity of TNTs remain conflicting. For instance, Fenyvesi and coworkers reported that in Caco-2 cells monolayer, TNTs are not cytotoxic [30]. In contrast, several studies have identified distinct rates of TNTs cytotoxicity particularly due to their inherent genotoxicity [31, 32]. It is also worth to mention that a few reports indicated that hydrothermally synthesised TiO_2 nanofilaments/nanorods are markedly cytotoxic to human epithelial cells [33, 34].

In addition, several studies have indicated that high aspect ratio (HAR) materials can cause profound cytotoxic effects, which are not observed when applying their 0-D forms [35, 36]. For instance, carbon NTs, as a well-known HAR material, can trigger macrophage (MΦ) activation resulting in a fibrosis [37]. In addition, other HAR nanomaterials, including Ce oxide nanorods [36], Ni [38] or Ag nanowires [39] also exhibit structure-dependent cytotoxicity. It is obvious that the cytotoxic activity HAR materials and underlying mechanisms are complex and are yet to be fully understood. Therefore, it is without a doubt that comprehensive nanotoxicological studies are essential to evaluate the potential cytotoxicity of HAR TiO_2 nanomaterials.

In the present study, we evaluate the cytotoxicity of bundles of TNT layers prepared *via* electrochemical anodization [10, 40], differing in the initial layer thickness. As far as we know, we provide the first data demonstrating that bundles of TNT layers exhibit inherent cell type-dependent cytotoxicity closely connected with their ability to disrupt the cell membrane integrity, followed by alteration of the morphometric parameters of cytoskeletal network and

dysregulation of intracellular redox homeostasis. Taken together, aside of an array of nanotoxicological data, the presented study provides an insight into the interesting properties of 1-D anodic TNT layers that could be highly promising for further development of advanced materials for nanomedicine.

2. Experimental Section

2.1. Chemicals

All chemicals were acquired from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified.

2.2. Preparation of self-organised TiO_2 nanotubes (TNT) layers and production of bundles

Self-organised TNT layers (inner diameter of ~110 nm and thickness of ~35 and ~10 μm) were synthesised by atomic layer deposition according to our previously published study [41]. Aging of the electrolyte (ethylene glycol based electrolyte containing 170 mM NH_4F and 1.5 vol% H_2O) was conducted in order to obtain different thicknesses while using the same type of electrolyte, as published previously [42]. A fresh electrolyte was used to produce ~35 μm thick TNT layers and an electrolyte used for 40 h was employed to produce ~10 μm thick TNT layers. After anodization, the TNT layers were sonicated in isopropanol and dried in air. To obtain ~35 and ~10 μm thick bundles of TNT layers (hereinafter referred to as TNTs-1 and TNTs-2, respectively), the TNT layers were mechanically bended and thus liberated off the substrate in bundles. The morphology of TNT layers was characterised using a field-emission scanning electron microscope (FE-SEM) JEOL 7500F, Tokyo, Japan. Prior to biological testing, TNTs were tested for endotoxins presence using Pierce™ LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher Scientific, Waltham, MA, USA). For every assay, TNTs were prepared as fresh stock solution in sterile phosphate-buffered saline (PBS, pH 7.4).

2.3. Cell cultures

Cell cultures utilised in the study were: *i*) the HEK-293 representing healthy human embryonic kidney cells and one of the most frequently used model in investigation of biological behaviour of nanomaterials, *ii*) the MDA-MB-231, triple-negative metastatic breast cancer cells exhibiting stem-cell like properties connected with pronounced chemoresistance, making these cells an interesting model for evaluation of cytotoxicity of novel nanomaterials, and *iii*) the RAW 264.7 murine MΦ. All cell cultures were acquired from American Type Culture Collection (Manassas, VA, USA). The culture media were as follows: DMEM for HEK-293 and RAW 264.7 MΦ, RPMI-1640 for MDA-MB-231 cells. In addition, RAW 264.7 cells were cultivated in 50/50% (*v/v*) fresh/conditioned DMEM.

2.4. Cytotoxicity screenings

The cytotoxic screenings were conducted using the XTT assay (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide) following previously published conditions [43].

2.5. Confocal reflectance microscopy (CRM)

After seeding the cells onto coverslips (~4×10^5 cells/coverslip) and overnight incubation the cells were administered with TNTs (125 μg/mL, 3 h). After incubation and washing using PBS, the cells were fixed in 4% formaldehyde (20 min, 25 °C). Then, the cells were permeabilised with 0.2% Triton X-100 (10 min), rinsed with PBS (3×) and blocked with 3% bovine serum albumin (BSA, 20 min) and labelled using Alexa Fluor 488-phalloidin (1:200) for 1 h at 37 °C to label the F-actin cytoskeletal network. Finally, the cells were rinsed using PBS, and the coverslips were mounted with ProLong™ Gold Antifade Mountant with 4´,6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific) to counterstain nuclei. To visualize TNTs by CRM (LSM 880, Carl Zeiss, Jena, Germany), the backscattered light from TNTs was collected within a detection window (559-564 nm) upon irradiation by a solid state 561 nm laser.

2.6. Cell membrane integrity - lactate dehydrogenase (LDH) leakage

Cells were seeded into 96-well plate (~10^5 cells/well) and left to adhere overnight. After treatment (125 μg/mL, 6 h), the LDH leakage was evaluated using the Pierce™ LDH Assay Kit (Thermo Fisher Scientific) following the instructions by the manufacturer and analysed using Infinite 200 PRO (Tecan). The leakage of LDH from TNTs-treated cells is expressed as % of the LDH released from fully lysed cells (positive control).

2.7. Cryo-FE-SEM

Cells were seeded onto carbon stubs (~10^5 cells/stub) and left to adhere overnight. After the treatment with TNTs (125 μg/mL, 6 h), samples were frozen in liquid nitrogen using PP3010 Cryo-SEM Preparation System (Quorum Technologies, Sussex, UK) and transferred to the SEM chamber. The samples were examined under high vacuum using MAIA3 SEM equipped with a field-emission gun (Tescan, Brno, Czech Republic). Images were obtained using the external SE detector at a working distance between 2.92-3.20 mm at 1 keV acceleration voltage.

2.8. Analysis of morphometric changes in F-actin microfilaments

Cells were seeded onto coverslips (~10^5 cells/coverslip) and cultured overnight. After that, the cells were administered with TNTs (125 μg/mL, 24 h). After treatment, the cells were rinsed using PBS and fixed in 4% formaldehyde (15 min, 25 °C). Then, staining and confocal imaging of F-actin cytoskeleton and nuclear counterstain was performed as described above in chapter *2.5*. Micrographs were acquired using LSM 880 (Carl Zeiss) at the AiryScan superresolution mode. The obtained confocal data were analysed using ZEN 2.3 (blue edition, Carl Zeiss) and morphometric parameters [F-actin density, coherency and nucleus-to-cytoplasm ratio (N/C)] were evaluated on 20 cells using the ImageJ software (National Institute of Health, Bethesda, MD, USA). Coherency was analysed according to [44]. Integrated density represents the total sum of the pixels in the regions of interests within the confocal micrographs.

2.9. Analysis of production of ROS

After seeding (~10^5 cells/well, 96-well plates), the cells were left to adhere overnight. After the treatment with TNTs (125 μg/mL, 12 h) and washing with culture media, the cells were stained using CellROX® Green Reagent following the instructions by the manufacturer. Nuclei were counterstained using Hoechst 33258. Samples were analysed using EVOS FL Auto Cell Imaging System (Thermo Fisher Scientific). Additionally, ROS stained according to the same protocol were quantified in 10^4 events using the BD Accuri C6 Plus flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

2.10. Analysis of DNA fragmentation using Comet assay

After seeding the cells (~10^6 cells/well) and exposing them to TNTs (125 μg/mL/12 h), DNA fragmentation was investigated using Comet assay performed according to Olive and coworkers [45]. The content of DNA fragmentation was quantified from fluorescence micrographs captured using the EVOS FL Auto Cell Imaging System (Thermo Fisher Scientific). The classification of comets was based on the length and the shape of the comet tails.

2.11. Analysis of lipid peroxidation

After seeding (~10^6 cells/well, six-well plate), the cells were administered with TNTs (125 μg/mL, 12 h). Induction of lipid peroxidation was studied using Image IT™ Lipid Peroxidation Kit (Thermo Fisher Scientific) following the instructions by manufacturer. The kit provides an oxidant inducer, cumenehydroperoxide (CHP), which was utilised at 100 μM. The stained cells were studied using the EVOS FL Auto Cell Imaging System (Thermo Fisher Scientific). Ratios of fluorescence intensity were calculated using the SimplePCI (Hamamatsu Corporation, Sewickley, PA, USA).

2.12. Quantitation of induction of apoptosis/necrosis

A suspension of 5×10^5 cells was incubated with TNTs (125 μg/mL, 12 h). Apoptosis and necrosis was evaluated in 10^4 events using the BD Accuri C6 Plus flow cytometer (BD

Biosciences) after staining with the PE Annexin V Apoptosis Detection Kit I (BD Biosciences). The induction of apoptosis and necrosis was further validated through cytopathological examination using May-Grünwald Giemsa (MGG) staining according to the previously published guidelines [46].

2.13. Western blotting

After the treatment with TNTs (125 µg/mL, 12 h), protein extraction was carried out using 100 µL of radioimmunoprecipitation buffer. Following the electrotransfer and blocking using 5% (*w/v*) skim milk powder (1 h at 37 °C), the proteins were immunolabelled using following primary antibodies: *i*) mouse anti-GAPDH (1:750, sc-365062, Santa Cruz Biotechnology, Dallas, TX, USA), *ii*) mouse anti-β-actin (1:2,000, ab8226, Abcam, Cambridge, UK), *iii*) mouse anti-Bcl-2 (1:250, sc7382, Santa Cruz Biotechnology), *iv*) mouse anti-p53 (1:250, sc126, Santa Cruz Biotechnology), *v*) mouse anti-MT1-1/2-E9 (1:200, M0639, Dako, Glostrup, Denmark), *vi*) mouse anti-MT-3 (1:700, LS-C197711, LSBio, Seattle, WA, USA) and *vii*) mouse procaspase-3 (1:250, ab136812, Abcam). Upon rinsing, the primary antibodies were immunolabelled with horseradish peroxidase-labelled secondary antibody (p0260, 1:5,000, Dako) for 1 h at 20 °C. After that, the chemiluminescence was developed with Clarity Western ECL Blotting Substrate (Bio-Rad).

2.14. Investigation of lysosomal membrane integrity using acridine orange (AO)

Overnight cultures of cells (~10^5) in cell culture dishes were treated with TNTs (125 µg/mL, 12 h). As controls, cells were exposed to 500 µM H_2O_2 (2 h) or to 100 µM chloroquine (3 h) inducing a permeabilization of lysosomal membranes and alkalinization of lysosomes, respectively. After rinsing using PBS, the cells were treated with metachromatic dye AO (10 µg/mL) and incubated at 37 °C for 20 min. To evaluate the red-to-green fluorescence intensity ratio (R/GFIR), the cells were rinsed with fresh medium and imaged using LSM 880 (Carl Zeiss) utilising λ_{exc} 488 nm and λ_{em} 493-550 nm or 590-720 nm. Data analysis was performed

using ImageJ (National Institute of Health). Quantitation of fluorescence was typically based on 20-30 cells per image with the results being normalised as fluorescence/cell.

2.15. Evaluation of interactions between TNTs and blood components

Haemocompatibility of TNTs was evaluated on commercially available human red blood cells (RBCs) (Zen-Bio, Durham, NC, USA) by adopting the protocol published in our previous study [47]. In addition, formation of protein coronas and activation of third complement component (C3) due to TNTs exposure *in vitro* were studied according to [48]. As a positive control for C3 activation analyses, we utilised dextran-coated superparamagnetic iron oxide nanoparticles (SPIONs) that are known to efficiently activate C3 [49].

2.16. Evaluation of RAW 264.7 MΦ polarization

MΦ were scraped and seeded (~10^5 cells/well, six-well plate). Then, the cells were treated with TNTs (125 μg/mL) for annotated time points. After washing the cells with PBS (3×), the overall morphological features of MΦ polarization (lamellipodial extensions, elongated bipolar morphology, enlarged amoeboid cell shape) were analysed using EVOS FL Auto Cell Imaging System (Thermo Fisher Scientific) at a phase contrast mode. For quantitation, the number of cells with typical morphological appearance out of at least 100 randomly selected cells was counted. As positive and negative controls, lipopolysaccharide (LPS, 5 ng/mL) and DMEM were utilised.

3. Results

3.1. Characterization of bundles of TNT layers (TNTs-1 and TNTs-2)

TNT layers were synthesised *via* electrochemical anodization of Ti foils according to our previous study [41]. **Fig. 1A-D** show TNT layers with an inner diameter of ~110 nm and a thickness of ~35 or ~10 μm, respectively. After mechanical bending of TNT layers and liberation of TNTs, it was found that the resulting ~35 and ~10 μm thick TNT bundles (TNTs-

1 and TNTs-2, respectively) dispersed in water exhibited the nanotubular morphology. **Fig. 1E**
2 and **1F** clearly show that bending of TNT layers lead to breakage of the layers into smaller
3 fragments - bundles - retaining the diameter of NTs. In addition, **Fig. 1F** demonstrates that the
4 incubation of TNTs in RPMI-1640 with 10% FBS resulted in a formation of layer equally
5 distributed on the TNTs surface.

6 *3.2. TNTs exhibit preferential in vitro cytotoxicity in malignant cells*

7 First, we screened the viability of cells upon administration with TNTs-1 and TNTs-2. None of
8 the tested TNTs significantly affected the viability of non-malignant HEK-293 cells (**Fig. 2A**).
9 In contrast, malignant MDA-MB-231 cells exhibited a dose-dependent reduction of viability.
10 The cytotoxicity markedly increased with a prolonged administration time (up to 72 h).
11 Moreover, increased time of exposure resulted in a significant (p<0.05 at 48 and 72 h time-
12 points) enhancement of cytotoxicity of TNTs-2 (**Fig. 2B**). CRM analysis upon a short-time (3
13 h) exposure revealed that both types of TNTs tended to reside within the cellular area delimited
14 by F-actin staining and except for TNTs, some portion of smaller fragments of tubes can be
15 found (**Fig. 2C**). Interestingly, **Fig. 2D** indicates that upon interaction with cell membranes,
16 both types of TNTs were capable of disrupting the cell membranes´ integrity. Significantly
17 (p<0.01) higher LDH leakage was found for MDA-MB-231 cells, in which TNTs-1 caused
18 more efficient leakage compared to TNTs-2 (p<0.05), which is in line with the cytotoxicity
19 screenings.

20 *3.3. TNTs are partially inserted into the cell membrane*

21 With respect to the obtained data, we performed additional cryo-FE-SEM analyses focused on
22 the visualization of the contact interface between TNTs-2 and tested cells. **Fig. 3A** indicates
23 that after 6 h treatment, TNTs-2 were partially inserted into the cell membrane and penetrated
24 to the intracellular region of MDA-MB-231 cells, while being loosely laid on the surface of
25 HEK-293 cells. Using the maximised depth of field of FE-SEM imaging, we found that at the

site of contact between the cell membrane and the TNTs-2, the cell membrane of MDA-MB-231 cells was partially covering the surface of penetrating TNTs-2 (**Fig. 3B**). Taken together, FE-SEM micrographs corroborated the outputs of membrane integrity analyses and highlighted that TNTs are capable of being inserted into the cell membranes.

3.4. TNTs cause rearrangement of F-actin cytoskeletal network

Based on the findings that TNTs are penetrating the cell membranes, we further focused on their effect on organization of cytoskeletal network. **Fig. 4A** shows that the control cells exhibited straight, well-arranged F-actin microfilaments. In contrast, upon treatment with TNTs-2, a disordered arrangement of F-actin fibres was evident, together with the accumulation of F-actin in the contact zone between the cell and the TNTs-2 (**Fig. 4B**). This phenomenon was further highlighted by the constructed 2.5-D intensity profile of confocal acquisition indicating that the highest intensity of F-actin (grey scale) co-localised with the presence of TNTs-2 (red) (**Fig. 4C**). Noteworthy, we found that the integrated density of F-actin remained unaffected upon TNTs treatments (**Fig. 4D**). In contrast, the evaluation of coherency confirmed disparate spatial reorganization of F-actin (**Fig. 4E**). The coherency values of TNTs-treated cells underwent a significant ($p<0.01$) decrease due to TNTs exposure. In addition, TNTs-2 that exhibit higher cytotoxicity caused a significant ($p<0.05$) decrease in F-actin coherency compared to TNTs-1. Overall, both kinds of TNTs induced a reorganization of the isotropic cytoskeletal network, but did not markedly affect the amount of F-actin. Additionally, **Fig. 4F** demonstrates that in line with the rearrangement of cytoskeletal network, the TNTs also induced a significant ($p<0.01$ for TNTs-2 and $p<0.05$ for TNTs-1) increase in N/C ratio.

3.5. Upon insertion, TNTs disrupt redox homeostasis, preferentially in malignant cells

To understand the intracellular events occurring as a follow-up of insertion of TNTs into cell membranes, we focused on redox homeostasis that is frequently disrupted by metal-based materials [50]. Interestingly, **Fig. 5A** shows that in MDA-MB-231 cells, both types of TNTs

induced pronounced formation of ROS; however, no ROS induction was found in HEK-293 cells. This phenomenon was confirmed by flow cytometry, corroborating that MDA-MB-231 cells were significantly (p<0.05 for TNTs-1 and p<0.01 for TNTs-2) more prone to TNTs-induced oxidative stress compared to HEK-293 cells (**Fig. 5B**). In line with ROS formation, TNTs were capable to induce lipid peroxidation (**Fig. 5C**), which was significantly (p<0.05 for TNTs-1 and p<0.01 for TNTs-2) elevated in MDA-MB-231 cells (**Fig. 5D**).

Because of disruption of redox homeostasis, pronounced DNA fragmentation was found in MDA-MB-231 cells treated with TNTs-2 (**Fig. 6A**). It is worth to note that both types of TNTs induced lower-graded comets (**Fig. 6B**). Therefore, we anticipate that longer administration of TNTs could result in a larger extent of DNA fragmentation.

3.6. TNTs induce apoptosis and cause lysosomal alkalinization

Since DNA fragmentation is a distinctive feature of apoptosis, next experiments were focused on the relation between TNTs treatment and a type of cell death. **Fig. 7A** summarizing apoptotic/necrotic events corroborates that MDA-MB-231 cells exhibit significantly (p<0.01) higher susceptibility to TNTs compared to HEK-293 cells. Moreover, the ability of TNTs-2 to induce a significantly (p<0.01 compared to TNTs-1) higher rate of apoptosis/necrosis was confirmed by cytopathological evaluation of MGG-stained cells shown in **Fig. 7B** (see membrane blebbing or spill out of cytoplasm). In addition, in MDA-MB-231 cells, both types of TNTs caused down-regulation of procaspase-3 and Bcl-2 highlighting the activation of the intrinsic execution-phase of apoptosis (**Fig. 7C**). Moreover, in HEK-293 cells, TNTs re-activated expression of MT-3, which was down-regulated in MDA-MB-231 cells. This phenomenon could be partially responsible for a higher resistance of HEK-293 cells to TNTs cytotoxicity. **Fig. 7D** shows that oxidative stress and apoptotic/necrotic cell death caused by TNTs was associated with alkalinization of lysosomes. In agreement with the rest of obtained

data, this phenomenon was significantly (p<0.01 for MDA-MB-231 cells, p<0.05 for HEK-293 cells) higher for TNTs-2 (**Fig. 7E**).

3.7. Evaluation of in vitro biocompatibility of TNTs

Next, we focused on interactions between TNTs and environment of blood circulation *in vitro*. As shown in **Fig. 8A**, both types of TNTs were capable of causing a concentration-dependent release of haemoglobin from human RBCs. Interestingly, only a negligible degree of formation of surface-adsorbed protein corona was identified indicating that both TNTs obviate the majority of interactions with plasma proteins (**Fig. 8B**). In agreement with insignificant surface protein coronas (frequently acting as opsonins), no activation of C3 by TNTs was identified (**Fig. 8C**). Interestingly, despite no C3 activation was found, both types of TNTs were recognised by MΦ and caused efficient polarization of their phenotypes (**Fig. 8D**). Noteworthy, TNTs-2 exposure resulted in a markedly faster polarization compared to administration with LPS (**Fig. 8E**), indicating their perspicuous effect on blood microenvironment.

4. Discussion

It is a general fact that cytotoxicity of nanomaterials is highly dependent on various aspects, such as dose (concentration and time of exposure), surface chemistry, particle morphology and type of cells [51]. Indeed, we demonstrated that cytotoxicity of both types of TNTs is dose- and time-dependent. Surprisingly, throughout the whole study, the cytotoxic effects of TNTs were significantly higher in MDA-MB-231 cells compared to their non-malignant (HEK-293) counterparts. This could be explained by the general fact that malignant cells exhibit markedly higher membrane fluidity and decreased rigidity affecting membrane permeability and endocytic functions [52] and also enhancing migratory/invasive abilities of cancer cells [53].

CRM analyses revealed that TNTs resided within the cellular region of both types of cells. However, cryo-FE-SEM revealed that in HEK-293 cells, majority of TNTs-2 are loosely laid on cells, while in MDA-MB-231 cells, TNTs-2 behave as spears passively penetrating the cell

membranes, which is a mechanism of internalization previously described for a number of nanotubular materials [54-56]. Importantly, we identified that even bigger non-spear-shaped TNTs are accumulated on cell membranes; however, only spear-shaped fragments were found penetrating the membranes, highlighting the importance of morphology of TNTs for interaction with cell membranes.

In perfect agreement with the membrane fluidity hypothesis, MDA-MB-231 cells were also more prone to TNTs-mediated disruption of membrane integrity as evidenced by significantly higher LDH leakage compared to HEK-293 cells. With respect to the obtained data, we further investigated the effects of TNTs on reorganization of the cytoskeletal network with a special emphasis on F-actin microfilaments, which play a critical role in pathophysiological architecture of cells [57]. In line with De Matteis and coworkers [58], who studied the morphometric parameters of cells upon administration with TiO_2 nanoparticles, our results revealed that despite TNTs F-actin density was not altered by TNTs treatment, F-actin coherency was significantly reduced due to exposure to TNTs-1 and TNTs-2, suggesting efficient reorganization of the cytoskeletal network upon insertion of TNTs. Furthermore, the evaluation of N/C ratios revealed that MDA-MB-231 cells underwent a marked increase of the cellular area through cytoplasm extension, a phenomenon previously linked to the cytotoxicity of multi-walled carbon NTs in cancer cells [59, 60].

To provide further insights into the follow-up intracellular mechanisms underlying the cytotoxic activity of TNTs, we focused on their effect on redox homeostasis, which is one of the hallmarks of cytotoxicity of inorganic nanomaterials, including those of TiO_2 origin. Indeed, we found an increased formation of free intracellular ROS and lipid peroxidation, corroborating the previously published studies [17, 27, 61]. In general, the excess in cellular levels of ROS causes an inevitable damage to the cellular structures and activates the signalling cascades leading to apoptosis [62]. Cytopathological examination revealed that in MDA-MB-231 cells,

TNTs-2 induce relatively high rate of necrosis. This could be explained by a differential efficiency of both types of TNTs to produce ROS. Lower amounts of ROS produced by TNTs-1 predominantly tended to induce apoptosis, whereas higher concentrations of ROS were also partially capable of inducing necrotic cell death, as has been demonstrated with H_2O_2 in HFL-1 cells [63].

In connection with these facts, we found that TNTs displayed the ability to alkalinize lysosomal lumen. Due to size of TNTs underpinning the obvious inability to be sequestered by endo-lysosomal compartments [64], most likely, elevation of intracellular ROS is responsible through oxidation-induced lysosomal alkalinization [65]. Importantly, the deactivation of lysosomes has a drastic impact on the cellular degradative capacity, which results in a wide spectrum of intracellular aberrations and may play a fundamental role in nanomaterial cytotoxicity [66].

Concerning the obtained data, two important points must be highlighted: *i*) TNTs-2 were pronouncedly more efficient in the dysregulation of redox homeostasis and the induction of apoptosis compared to TNTs-1, and *ii*) this phenomenon was prototypical for MDA-MB-231 cells. Taken together, both points are in line with the rest of the presented data and suggest that a passive penetration of TNTs into cell membranes is crucial to trigger the follow-up intracellular events leading to cell death.

Active surface chemistry, crystalline phase composition or distinct catalytic activities of nanomaterials have a dramatic impact on the resulting cytotoxicity [17, 67]. However, since both types of tested TNTs were prepared by the same anodization process, the influence of surface chemistry/crystallinity/differences in catalytic processes on higher cytotoxicity of TNTs-2 can be excluded. It must be pointed out that the presented data are contradictory with reports showing that the cytotoxicity of NTs positively correlates with their initial length. However, the fact that none of these reports evaluated a length-dependent cytotoxicity of TNTs and majority of them focused on carbon-based NTs [68-70] that can differ in wide spectrum of

properties (membrane affinity, medium stability, enzymomimetic activity, *etc*.) makes our results incomparable with current literature. Moreover, CRM indicates that together with TNTs, some portion of smaller fragments is present within the cellular area. These could likely contribute to cytotoxicity of TNTs. Hopefully, follow-up studies will shed light on the inverse phenomenon of TNTs length-dependent cytotoxicity.

It is also worth to note that using TNTs as a nanotoxicological model, we did not validate the findings by Wang *et al*. that in HAR materials, the aspect ratio positively correlates with material cytotoxicity as demonstrated for anodic alumina NTs [71]. Nevertheless, this phenomenon is most likely due to substantial differences between chemical composition and morphological features of tested materials.

Consistent with previous report by Horvath *et al*. [72], RAW 264.7 MΦ are highly susceptible to nanomaterials exposure. Both types of TNTs exhibited highly stimulatory effects on MΦ polarization rate. However, to further understand the possible pro- or anti-inflammatory effects of TNTs, future studies might be focused on analyses of the traditional dichotomy of MΦ polarization (M1 *vs*. M2) and subsequent release of cytokines [73]. In a contradiction to the fast polarization rate induced by TNTs, no activation of C3 by TNTs was identified, suggesting that the MΦ polarization by TNTs is triggered without the need of C3 activation [74]. Overall, both TNTs displayed a considerably undesired effect on cells representing blood environment and immune system. Unfortunately, there is a lack of available literature on immuno- and haematotoxicity of anodic TNTs, making the obtained results difficult to discuss.

Collectively, the findings presented in this study could be of utmost interest for a rational design of intelligent anticancer nanomedicines. Nevertheless, to fully prove this phenomenon, additional engineering towards producing uniform single anodic TiO_2 NTs and their comprehensive testing on a broad panel of cancer and healthy cell lines might be done. We are keen to further work on this aspect.

5. Conclusion

We clearly describe the comprehensive mechanisms underlying the cytotoxic activity of HAR bundles of TNT layers in human epithelial cells *in vitro*. We report on the capability of TNTs to penetrate cell membranes and to cause subsequent disruption of membrane integrity, followed by a cascade of events resulting in DNA fragmentation and apoptotic/necrotic cell death. Likewise, an array of *in vitro* biocompatiblity assays revealed that TNTs are harmful for RBCs and manifest potential immunogenicity. It is worth to note that throughout the whole study, both types of TNTs displayed significantly higher and selective cytotoxicity for malignant (MDA-MB-231) cells. We hypothesize that this phenomenon might be due to enhanced membrane fluidity and decreased rigidity; however, it must be noted that such differences could be also affected by a cell type (kidney *vs*. breast epithelia) or even culture conditions (DMEM *vs*. RPMI-1640). Despite these limitations, it is undoubtful that the description of the fundamental TNTs cytotoxicity paradigm in cancer cells offers further opportunities for a future engineering (tuning of size or surface chemistry) of highly sophisticated rational cancer nanomedicines exploiting differences in cell responses to TNTs.

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Conflict of interest

The authors declare no conflicts of interest.

References

1. D'Mello, S.R., et al., *The evolving landscape of drug products containing nanomaterials in the United States.* Nat. Nanotechnol., 2017. **12**(6): p. 523-529.
2. Maine, E., et al., *The emergence of the nanobiotechnology industry.* Nat. Nanotechnol., 2014. **9**(1): p. 2-5.
3. Mahmood, J., et al., *An efficient and pH-universal ruthenium-based catalyst for the hydrogen evolution reaction.* Nat. Nanotechnol., 2017. **12**(5): p. 441-446.
4. Chen, X. and S.S. Mao, *Titanium dioxide nanomaterials: Synthesis, properties, modifications, and applications.* Chem. Rev., 2007. **107**(7): p. 2891-2959.
5. He, W.Y., et al., *Plasmonic titanium nitride nanoparticles for in vivo photoacoustic tomography imaging and photothermal cancer therapy.* Biomaterials, 2017. **132**: p. 37-47.
6. Shi, X., et al., *Enhanced water splitting under modal strong coupling conditions.* Nat. Nanotechnol., 2018. **13**(10): p. 953-958.
7. Bayat, N., et al., *Vascular toxicity of ultra-small TiO2 nanoparticles and single walled carbon nanotubes in vitro and in vivo.* Biomaterials, 2015. **63**: p. 1-13.
8. Wang, X.D., et al., *One-Dimensional Titanium Dioxide Nanomaterials: Nanowires, Nanorods, and Nanobelts.* Chem. Rev., 2014. **114**(19): p. 9346-9384.
9. Dvorak, F., et al., *One-dimensional anodic TiO2 nanotubes coated by atomic layer deposition: Towards advanced applications.* Appl. Mater. Today, 2019. **14**: p. 1-20.
10. Macak, J.M., et al., *TiO2 nanotubes: Self-organized electrochemical formation, properties and applications.* Curr. Opin. Solid State Mat. Sci., 2007. **11**(1-2): p. 3-18.
11. Albu, S.P., et al., *Self-organized, free-standing TiO2 nanotube membrane for flow-through photocatalytic applications.* Nano Lett., 2007. **7**(5): p. 1286-1289.
12. Riboni, F., et al., *Aligned metal oxide nanotube arrays: key-aspects of anodic TiO2 nanotube formation and properties.* Nanoscale Horiz., 2016. **1**(6): p. 445-466.
13. Kupcik, R., et al., *New Interface for Purification of Proteins: One-Dimensional TiO2 Nanotubes Decorated by Fe3O4 Nanoparticles.* ACS Appl. Mater. Interfaces, 2017. **9**(34): p. 28233-28242.
14. Kupcik, R., et al., *Amorphous TiO2 Nanotubes as a Platform for Highly Selective Phosphopeptide Enrichment.* ACS Omega, 2019. **4**(7): p. 12156-12166.
15. Gulati, K., et al., *Biocompatible polymer coating of titania nanotube arrays for improved drug elution and osteoblast adhesion.* Acta Biomater., 2012. **8**(1): p. 449-456.
16. Malysheva, A., E. Lombi, and N.H. Voelcker, *Bridging the divide between human and environmental nanotoxicology.* Nat. Nanotechnol., 2015. **10**(10): p. 835-844.
17. Skubalova, Z., et al., *Prevalent anatase crystalline phase increases the cytotoxicity of biphasic titanium dioxide nanoparticles in mammalian cells.* Colloids Surf. B, 2019. **182**: p. 1-10.
18. Jin, C.Y., et al., *Cytotoxicity of titanium dioxide nanoparticles in mouse fibroblast cells.* Chem. Res. Toxicol., 2008. **21**(9): p. 1871-1877.
19. Sha, B.Y., et al., *Cytotoxicity of titanium dioxide nanoparticles differs in four liver cells from human and rat.* Compos. Pt. B-Eng., 2011. **42**(8): p. 2136-2144.
20. Wang, Y.R., et al., *Cytotoxicity, DNA damage, and apoptosis induced by titanium dioxide nanoparticles in human non-small cell lung cancer A549 cells.* Environ. Sci. Pollut. Res., 2015. **22**(7): p. 5519-5530.
21. Ma, Y., et al., *Titanium dioxide nanoparticles induce size-dependent cytotoxicity and genomic DNA hypomethylation in human respiratory cells.* RSC Adv., 2017. **7**(38): p. 23560-23572.
22. Wang, Y.L., et al., *Excess titanium dioxide nanoparticles on the cell surface induce cytotoxicity by hindering ion exchange and disrupting exocytosis processes.* Nanoscale, 2015. **7**(30): p. 13105-13115.

23. Wei, Z.C., et al., *Effect of particle size on in vitro cytotoxicity of titania and alumina nanoparticles.* J. Exp. Nanosci., 2014. **9**(6): p. 625-638.
24. Verma, S.K., et al., *Mechanistic Insight into Size-Dependent Enhanced Cytotoxicity of Industrial Antibacterial Titanium Oxide Nanoparticles on Colon Cells Because of Reactive Oxygen Species Quenching and Neutral Lipid Alteration.* ACS Omega, 2018. **3**(1): p. 1244-1262.
25. Sohaebuddin, S.K., et al., *Nanomaterial cytotoxicity is composition, size, and cell type dependent.* Part. Fibre Toxicol., 2010. **7**: p. 1-17.
26. Xu, Y., et al., *Cell-based cytotoxicity assays for engineered nanomaterials safety screening: exposure of adipose derived stromal cells to titanium dioxide nanoparticles.* J. Nanobiotechnol., 2017. **15**: p. 1-17.
27. Ahamed, M., et al., *Ag-doping regulates the cytotoxicity of TiO2 nanoparticles via oxidative stress in human cancer cells.* Sci. Rep., 2017. **7**: p. 1-14.
28. Wang, N., et al., *Effects of TiO2 nanotubes with different diameters on gene expression and osseointegration of implants in minipigs.* Biomaterials, 2011. **32**(29): p. 6900-6911.
29. Su, E.P., et al., *Effects of titanium nanotubes on the osseointegration, cell differentiation, mineralisation and antibacterial properties of orthopaedic implant surfaces.* Bone Joint J., 2018. **100B**(1): p. 9-16.
30. Fenyvesi, F., et al., *Investigation of the Cytotoxic Effects of Titanate Nanotubes on Caco-2 Cells.* AAPS PharmSciTech, 2014. **15**(4): p. 858-861.
31. Mohamed, M.S., et al., *Anodically Grown Titania Nanotube Induced Cytotoxicity has Genotoxic Origins.* Sci. Rep., 2017. **7**: p. 1-11.
32. Chakrabarti, S., et al., *Exploration of cytotoxic and genotoxic endpoints following sub-chronic oral exposure to titanium dioxide nanoparticles.* Toxicol. Ind. Health, 2019. **35**(9): p. 577-592.
33. Magrez, A., et al., *Cellular Toxicity of TiO2-Based Nanofilaments.* ACS Nano, 2009. **3**(8): p. 2274-2280.
34. Chen, J.Y., et al., *Evaluating Cytotoxicity and Cellular Uptake from the Presence of Variously Processed TiO2 Nanostructured Morphologies.* Chem. Res. Toxicol., 2010. **23**(5): p. 871-879.
35. Huang, X.L., et al., *The effect of the shape of mesoporous silica nanoparticles on cellular uptake and cell function.* Biomaterials, 2010. **31**(3): p. 438-448.
36. Lin, S.J., et al., *Aspect Ratio Plays a Role in the Hazard Potential of CeO2 Nanoparticles in Mouse Lung and Zebrafish Gastrointestinal Tract.* ACS Nano, 2014. **8**(5): p. 4450-4464.
37. Wang, P., et al., *Multiwall carbon nanotubes mediate macrophage activation and promote pulmonary fibrosis through TGF-beta/Smad signaling pathway.* Small, 2013. **9**(22): p. 3799-811.
38. Poland, C.A., et al., *Length-dependent pathogenic effects of nickel nanowires in the lungs and the peritoneal cavity.* Nanotoxicology, 2012. **6**(8): p. 899-911.
39. Schinwald, A. and K. Donaldson, *Use of back-scatter electron signals to visualise cell/nanowires interactions in vitro and in vivo; frustrated phagocytosis of long fibres in macrophages and compartmentalisation in mesothelial cells in vivo.* Part. Fibre Toxicol., 2012. **9**: p. 1-13.
40. Lee, K., A. Mazare, and P. Schmuki, *One-Dimensional Titanium Dioxide Nanomaterials: Nanotubes.* Chem. Rev., 2014. **114**(19): p. 9385-9454.
41. Zazpe, R., et al., *Atomic Layer Deposition for Coating of High Aspect Ratio TiO2 Nanotube Layers.* Langmuir, 2016. **32**(41): p. 10551-10558.
42. Sopha, H., et al., *Effect of electrolyte age and potential changes on the morphology of TiO2 nanotubes.* J. Electroanal. Chem., 2015. **759**: p. 122-128.

43. Moulick, A., et al., *Real-Time Visualization of Cell Membrane Damage Using Gadolinium-Schiff Base Complex-Doped Quantum Dots.* ACS Appl. Mater. Interfaces, 2018. **10**(42): p. 35859-35868.
44. Clemons, T.D., et al., *Coherency image analysis to quantify collagen architecture: implications in scar assessment.* RSC Adv., 2018. **8**(18): p. 9661-9669.
45. Olive, P.L., et al., *The comet assay: A new method to examine heterogeneity associated with solid tumors.* Nat. Med., 1998. **4**(1): p. 103-105.
46. Piaton, E., et al., *Guidelines for May-Grunwald-Giemsa staining in haematology and non-gynaecological cytopathology: recommendations of the French Society of Clinical Cytology (SFCC) and of the French Association for Quality Assurance in Anatomic and Cytologic Pathology (AFAQAP).* Cytopathology, 2016. **27**(5): p. 359-368.
47. Buchtelova, H., et al., *Improving cytocompatibility of CdTe quantum dots by Schiff-base-coordinated lanthanides surface doping.* J. Nanobiotechnol., 2018. **16**: p. 1-14.
48. Michalkova, H., et al., *Tuning the surface coating of IONs toward efficient sonochemical tethering and sustained liberation of topoisomerase II poisons.* Int. J. Nanomed., 2019. **14**(1): p. 55-66.
49. Chen, F.F., et al., *Complement proteins bind to nanoparticle protein corona and undergo dynamic exchange in vivo.* Nat. Nanotechnol., 2017. **12**(4): p. 387-393.
50. Stone, V. and K. Donaldson, *Nanotoxicology - Signs of stress.* Nat. Nanotechnol., 2006. **1**(1): p. 23-24.
51. Krug, H.F. and P. Wick, *Nanotoxicology: An Interdisciplinary Challenge.* Angew. Chem.-Int. Edit., 2011. **50**(6): p. 1260-1278.
52. Peetla, C., S. Vijayaraghavalu, and V. Labhasetwar, *Biophysics of cell membrane lipids in cancer drug resistance: Implications for drug transport and drug delivery with nanoparticles.* Adv. Drug Deliv. Rev., 2013. **65**(13-14): p. 1686-1698.
53. Zhao, W.N., et al., *Candidate Antimetastasis Drugs Suppress the Metastatic Capacity of Breast Cancer Cells by Reducing Membrane Fluidity.* Cancer Res., 2016. **76**(7): p. 2037-2049.
54. Kraszewski, S., et al., *How long a functionalized carbon nanotube can passively penetrate a lipid membrane.* Carbon, 2012. **50**(14): p. 5301-5308.
55. Jachak, A., et al., *Transport of metal oxide nanoparticles and single-walled carbon nanotubes in human mucus.* Nanotoxicology, 2012. **6**(6): p. 614-622.
56. Lin, J.Q., et al., *Penetration of Lipid Membranes by Gold Nanoparticles: Insights into Cellular Uptake, Cytotoxicity, and Their Relationship.* ACS Nano, 2010. **4**(9): p. 5421-5429.
57. Stossel, T.P., et al., *Filamins as integrators of cell mechanics and signalling.* Nat. Rev. Mol. Cell Biol., 2001. **2**(2): p. 138-145.
58. De Matteis, V., et al., *Tailoring Cell Morphomechanical Perturbations Through Metal Oxide Nanoparticles.* Nanoscale Res. Lett., 2019. **14**: p. 1-18.
59. Rodriguez-Fernandez, L., et al., *Multiwalled Carbon Nanotubes Display Microtubule Biomimetic Properties in Vivo, Enhancing Microtubule Assembly and Stabilization.* ACS Nano, 2012. **6**(8): p. 6614-6625.
60. Gonzalez-Lavado, E., et al., *Biodegradable multi-walled carbon nanotubes trigger anti-tumoral effects.* Nanoscale, 2018. **10**(23): p. 11013-11020.
61. Xia, T., et al., *Comparison of the abilities of ambient and manufactured nanoparticles to induce cellular toxicity according to an oxidative stress paradigm.* Nano Lett., 2006. **6**(8): p. 1794-1807.
62. Redza-Dutordoir, M. and D.A. Averill-Bates, *Activation of apoptosis signalling pathways by reactive oxygen species.* Biochim. Biophys. Acta-Mol. Cell Res., 2016. **1863**(12): p. 2977-2992.

63. Vairetti, M., et al., *Apoptosis vs. necrosis: glutathione-mediated cell death during rewarming of rat hepatocytes.* Biochim. Biophys. Acta-Mol. Basis Dis., 2005. **1740**(3): p. 367-374.
64. Sahay, G., D.Y. Alakhova, and A.V. Kabanov, *Endocytosis of nanomedicines.* J. Control. Release, 2010. **145**(3): p. 182-195.
65. Pascua-Maestro, R., et al., *Protecting cells by protecting their vulnerable lysosomes: Identification of a new mechanism for preserving lysosomal functional integrity upon oxidative stress.* PLoS Genet., 2017. **13**(2): p. 1-33.
66. Stern, S.T., P.P. Adiseshaiah, and R.M. Crist, *Autophagy and lysosomal dysfunction as emerging mechanisms of nanomaterial toxicity.* Part. Fibre Toxicol., 2012. **9**: p. 1-16.
67. Qiu, Y., et al., *Surface chemistry and aspect ratio mediated cellular uptake of Au nanorods.* Biomaterials, 2010. **31**(30): p. 7606-7619.
68. Di Cristo, L., et al., *Comparative in Vitro Cytotoxicity of Realistic Doses of Benchmark Multi-Walled Carbon Nanotubes towards Macrophages and Airway Epithelial Cells.* Nanomaterials, 2019. **9**(7): p. 1-17.
69. Liu, D., et al., *Different cellular response mechanisms contribute to the length-dependent cytotoxicity of multi-walled carbon nanotubes.* Nanoscale Res. Lett., 2012. **7**: p. 10.
70. Shen, Z.Y., et al., *Comparison of cytotoxicity and membrane efflux pump inhibition in HepG2 cells induced by single-walled carbon nanotubes with different length and functional groups.* Sci. Rep., 2019. **9**: p. 1-9.
71. Wang, Y., et al., *Systematic in vitro nanotoxicity study on anodic alumina nanotubes with engineered aspect ratio: Understanding nanotoxicity by a nanomaterial model.* Biomaterials, 2015. **46**: p. 117-130.
72. Horvath, L., et al., *In Vitro Investigation of the Cellular Toxicity of Boron Nitride Nanotubes.* ACS Nano, 2011. **5**(5): p. 3800-3810.
73. Gustafson, H.H., et al., *Nanoparticle uptake: The phagocyte problem.* Nano Today, 2015. **10**(4): p. 487-510.
74. Pondman, K.M., et al., *Interactions of the innate immune system with carbon nanotubes.* Nanoscale Horiz., 2017. **2**(4): p. 174-186.

Figure Legends

Figure 1

Morphology of TiO_2 nanotube (TNT) layers. FE-SEM micrographs of (**A**) top and (**B**) cross-section of ~35 μm thick TNT layers (TNTs-1), and (**C**) top and (**D**) cross-section of ~10 μm thick TNT layers (TNTs-2). TNTs-1 and TNTs-2 were further dispersed in (**E**) MilliQ water or (**F**) RPMI-1640 supplemented with 10% FBS and their morphology was investigated by FE-SEM after 12 h storage at ambient conditions.

Figure 2

Cytotoxic activity and uptake of bundles of TiO_2 nanotubes (TNTs). (**A**) XTT assay showing HEK-293 and MDA-MB-231 cells after treatment with TNTs at various concentrations (0.49-500.00 μg/mL, 24 h). (**B**) XTT assay showing the viability of tested cells upon separate incubations with 125 μg/mL of TNTs at various time intervals (24, 48, or 72 h, respectively). (**C**) CRM of uptake of TNTs (125 μg/mL, 3 h). Images depict maximum intensity Z-projections of cells stained with DAPI nuclear counterstaining (blue), F-actin stained with FITC-labelled phalloidin (green) and TNTs reflection spots (red). Scale bar, 20 μm. (**D**) LDH assay showing leakage of LDH due to altered membrane integrity. The data represent three separate experiments (n = 3) and are expressed as mean values ± SD. *p<0.05, **p<0.01 *vs*. a control (non-treated) group.

Figure 3

Bundles of TiO_2 nanotubes (TNTs) are inserted into the plasma membrane. (**A**) Representative cryo-FE-SEM micrographs indicating insertion of ~10 μm thick bundles of TNTs-2 into membrane of both types of tested cells. Scale bars, 10 μm. (**B**) The contact interface between MDA-MB-231 cells and TNTs-2 was further studied using the depth display mode for imaging of samples with complex topography. Scale bars, 1 μm.

Figure 4

Bundles of TiO_2 nanotubes (TNTs-1 and TNTs-2, respectively) cause remodelling of F-actin cytoskeletal network. (**A**) Representative confocal micrographs of MDA-MB-231 cells exposed to TNTs-2 for 24 h; successively fixed and visualised for F-actin cytoskeletal network and reflectance of TNTs-2. Scale bar, 10 µm. (**B**) Local enlargement of confocal acquisition showing F-actin microfilaments accumulation in the zone of contact with TNTs-2. Scale bar, 1 µm. (**C**) 2.5-D intensity profile of confocal micrograph indicating association between TNTs-2 presence (red) and higher fluorescence intensity of F-actin (grey scale). (**D**) Integrated density and (**E**) coherency of F-actin for MDA-MB-231 cells treated with TNTs-1 and TNTs-2. (**F**) N/C ratio of MDA-MB-231 cells exposed to TNTs-1 and TNTs-2. Values are expressed as mean values ± SD, calculated from confocal micrographs by ImageJ (calculation on 20 cells). *p<0.05, **p<0.01 *vs*. a control (non-treated) group.

Figure 5

In MDA-MB-231 cells, bundles of TiO_2 nanotubes (TNTs) cause DNA damage through induction of oxidative stress. (**A**) Fluorescence microscopy of intracellular ROS formation in HEK-293 and MDA-MB-231 cells analysed using CellROX Green reagent (green). Blue, nuclei counterstain (Hoechst 33258). Scale bar, 30 µm. (**B**) The quantitation of ROS production. The fluorescence intensity of CellROX Green reagent was analysed using flow cytometry. (**C**) Representative micrographs of lipid peroxidation imaging, scale bar 50 µm. (**D**) Quantitation of fluorescence intensities of lipid peroxidation presented as a ratio of 590/510 nm. CHP, cumene hydroperoxide. Data represent three separate experiments (n = 3) and are expressed as mean values ± SD. *p<0.05, **p<0.01 *vs*. a control (non-treated) group.

Figure 6

TNTs exposure results in DNA fragmentation. (**A**) SCGE micrographs showing TNTs-induced DNA fragmentation prevalent in MDA-MB-231 cells. Scale bar, 50 µm. (**B**) Bar graphs shows quantitation of SCGE-based index of damage (comet grades) upon 12 h exposure to TNTs.

Figure 7

Bundles of TiO_2 nanotubes (TNTs) trigger apoptosis and necrosis in MDA-MB-231 cells. (**A**) Distribution of apoptosis/necrosis in cells exposed to 125 µg/mL of TNTs for 6 h quantified by flow cytometry after Annexin V-FITC/7-AAD staining. (**B**) MGG stained cytopathological preparations showing abundant presence of apoptotic and necrotic morphological features in MDA-MB-231 cells. Red arrowhead indicates apoptosis, green necrosis. Scale bar, 100 µm. (**C**) Immunoblots and densitometric analysis (values below blots) showing the influence of TNTs on expression of selected proteins. β-actin and GAPDH, loading controls. (**D**) Evaluation of lysosomal stability using AO staining. Cells treated with chloroquine and H_2O_2 served as positive controls of lysosomal alkalinization and lysosomal membrane permeabilization, respectively. Confocal micrographs show the R/GFIR of drops of AO. Scale bar, 30 µm. (**E**) Quantitation of red and green fluorescence performed using ImageJ. The data represent three separate experiments (n = 3) and are expressed as mean values ± SD. *p<0.05, **p<0.01 *vs*. a control (non-treated) group.

Figure 8

Evaluation of *in vitro* biocompatibility of bundles of TiO_2 nanotubes (TNTs). (**A**) Haemolysis investigated using human RBCs. Upper images show real photographs of analysed specimens. The values are expressed as the mean of three (n = 3) independent replicates ± SD. *p<0.05, **p<0.01 *vs*. negative control (PBS) group. (**B**) Gels showing the eluted protein coronas formed upon 60 min treatment of human plasma with TNTs. (**C**) Immunoblot of opsonization of TNTs by C3. Dextran-coated SPIONs were utilised as positive control. (**D**) Representative phase contrast micrographs of RAW 264.7 MΦ incubated with or without TNTs, scale bar 50 µm.

Micrographs indicate fast onset of typical morphological features of MΦ activation, which were quantified and are shown in bar graph (**E**). The data represent three separate experiments (n = 3) and are expressed as mean values ± SD.