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Cytotoxicity of Titanium and Silicon Dioxide Nanoparticles

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Abstract. Different TiO_2 and SiO_2 nanoparticles have been tested concerning their toxicity on selected mammalian cell lines. Various powders and suspensions, all of which consist of titanium or silicon dioxide nanoparticles have been examined. These particles differ in the crystal structure, the size and the BET-surface area. There was also a classification in fixed particles and in particles easily accessible in solution. With focus on the possible adsorption of the nanoparticles into the human organism, via skin and via respiratory tract, the effects on fibroblasts (NIH-3T3) and on a human lung adenocarcinoma epithelial cell line were examined. Additionally, the particles were tested with HEP-G2 cells, which are often used as model cell line for biocompatibility tests, and PC-12 cells, a rat adrenal pheochromocytoma cell line.

The viability of the cells was examined by the MTT-test. The viability results were found to partly depend on the type of cells used. The experimental results show that the adhesion of the cells on the different powders strongly depends on the type of cell lines as well as on the type of powder. It was found that the lower viability of some cells on the powder coatings is not only caused by a cytotoxicity effect of the powders, but is also due to a lower adhesion of the cells on the particle surfaces. Furthermore, it could be shown that the physical properties of the powders cannot be easily correlated to any observed biological effect. While some powders show a significant suppression of the cell growth, others with similar physical properties indicate no toxic effect.

1. Introduction

The technological progress allows the assembly of new, tiny material structures having the size of only a few nanometers. In this range the materials often exhibit different physical and chemical properties as compared with their bulk counterparts and can thus be employed for specific applications. Frequently used in its nanosize is titanium dioxide (TiO_2), *e.g.* in sun creams and toothpaste. A further growing field of application for TiO_2 is photocatalysis. In this area, titanium dioxide is; for example, used for self-cleaning surfaces as well as for the treatment of polluted water or air. SiO_2 nanoparticles are used, for example, as an anti-clumping-agent in common salt and as an additive in ketchup to decrease the adhesiveness. Due to the frequent use of these materials, it is important to confirm that their biological harmlessness still exists when prepared with particle sizes in the nanometer regime.

The environmental impact of as well as the human exposure to nanoparticles is currently being widely discussed. Presently, the exposure to mostly nanomaterials at the workplace is not regulated by maximum allowable concentration (MAC) -values. One important point for any risk analysis of nanotechnological products is the mobility of these nanoparticles. Depending on their manufacturing and application, nanoparticles can eventually reach the air or the ground water.

Most nanoparticles can bind large quantities of pollutants because of their larger surface area. Therefore, more toxic substances can be transported to the ground, including excrements and pesticides, which under normal conditions are terminally mobile. Because of the high reactivity of nanomaterials, it has even been reported that naturally nontoxic occurring substances can be transformed into toxic compounds. Because of the proven bactericidal effect of some materials, the microbial composition in the ground water could thus be changed and these particles could even be absorbed by plants and living organisms. Hence, they can enter the food chain resulting in an exposure of animals and humans. In different studies the toxic effect of various nanoparticles on living organisms in aquifers has therefore been investigated. For example, an increased mortality of *daphnia magna* after the exposition to titanium dioxide nanoparticles and fullerenes (C_{60}) was observed [1]. In water containing fullerenes at a concentration of 5 ppm a significant brain damage of Juvenile Largemouth Bass was detected by the measurement of lipid peroxidation and protein oxidation. Furthermore, inflammation of the liver was observed and taken as evidence for the diffusion of fullerenes into the whole body [2, 3]. Carbon Nanotubes diminish the procreation capability of zebra fish. Hundt-Rinke *et al.* [4] have investigated the algae growth upon addition of photocatalytically active titanium dioxide under illumination with UV light. It could be shown that nanoparticles exhibit an ecotoxicologic effect depending on their dimension and crystal structure [4]. Experiments with alumina nanoparticles indicated a reduced root growth on different useful plants such as maize, cucumber, soya and carrots. Exposition to larger particles, on the other hand, had no effect [5].

There are three possible absorption paths of nanoparticles into the human body: via the skin, via the respiratory chain and via the gastrointestinal tract. By oral assimilation, nanoparticles can be transported from the intestinal tract into the lymph stream and then into the blood [6]. By breathing, the particles can reach the lung and may cause inflammatory reactions because of their insufficient elimination by macrophages [7]. Carbon Nanotubes (CNT`s), for example, can cause such reactions in lung tissue [8, 9, 10]. An additional difficulty is the elimination of fibres. Animal tests employing rats have shown a direct absorption of nanoparticles from the nose to the brain, the potential risks of which have not yet been determined adequately [11]. Once they have been incorporated into the organism, nanoparticles can reach numerous different areas of the body because of their high mobility. Nanostructured substances can pass through biological barriers such as the blood-brain barrier or cell membranes. Moreover, they can move along the nerve pathways and arrive at organs like liver and kidney. The transfer of nanoparticles from the placenta into the foetus is not only a chance for a selective therapy use but it can also be a risk. However, the potential risk of such interactions is still widely unknown. The distribution of nanoparticles within the organism depends on their dimension, form, and material properties.

Bio-degradable nanoparticles are mostly harmless, but little is presently known concerning the fate of non bio-degradable particles [12]. It can be assumed that nanoparticles concentrate in the detoxification organs such as liver and kidney. In this case the definition of the respective MAC-values will be important.

Investigations employing human lung cells (A-549) which were exposed to different concentrations of Single Wall Carbon Nanotubes (SWCNT´s) showed a very low acute toxicity effect. Transmission electron microscopy (TEM) studies confirmed that there was no intracellular localization of SWCNT´s in A-549 cells following 24 h exposure, however, an increased number of surfactant storing lamellar bodies was observed in exposed cells [13].

The role of particle size and surface chemistry for the initiation of pro-inflammatory effects in human lung epithelial cells (A-549) after treatment with different TiO_2 particles was also investigated. TiO_2 particles were rapidly taken up by the cells, however, no particles were observed inside the

nuclei or any other vital organelle. TiO_2 particles even in the form of aggregates/agglomerates can trigger inflammatory responses that appear to be driven by their large surface area. It was suggested that these effects may result from oxidants generated during particle-cell interactions through an unknown mechanism [14].

In this study, nine different powders based on titanium dioxide nanoparticles and three different SiO_2 nanoparticulate preparations were investigated concerning their toxicity on four different mammalian cell lines. The particles differ in their crystal structure, their size, and their BET-surface area. Furthermore, there was a classification into fixed particles, coatings, and into particles easily accessable in their suspensions. Because nanoparticles can be incorporated into the organism via the skin, via the respiratory and via the alimentary system, the potential toxicity of the particles was investigated employing a human lung adenocarcinoma epithelial cell line (A-549), fibroblasts (NIH-3T3), a rat adrenal pheochromocytoma cell line (PC-12), and a human hepatocellular cell line (HEP-G2). The viability of the cell was examined by the MTT-test.

2. Materials and Methods

All solutions were prepared with deionised water (Arium, SARTORIUS, Göttingen, Germany). DMEM was purchased from Sigma-Aldrich (Steinheim, Germany) and foetal calf serum (FCS) and newborn calf serum from PAA (Cölbe, Germany). Horse serum (HOS) was purchased from Invitrogen (Karlsruhe, Germany).

Aeroxide TiO_2 P 25 was a kind gift from Evonik Industries (Hanau, Germany). PC 500, PC 100, PC 50, and PC 10 were a kind gift from Millennium Inorganic Chemicals (Grimsby, Great Britain) who also kindly supplied R 15, R 25, and R 34. The Silicon Dioxide Particles were synthesized by the working group of Prof. Peter Behrens from the Institute of Inorganic Chemistry in Hannover. Buffers, salts, antibiotics and other reagents were purchased from Fluka (Buchs, Switzerland) and Sigma-Aldrich (Steinheim, Germany) and are of *per analysi* quality.

2.1. Cell Culture

All cell lines were cultivated in Dulbecco's Modified Eagle's medium (DMEM) containing the according serum and 1 % antibiotics (penicillin and streptomycin) at 37°C / 5% CO_2. After 3 to 4 days the cells had grown to confluence and were then detached with trypsin and cultured in a new cell culture flask.

Table 1 shows the different DMEM additives as well as the cell number per well for the experiments.

Table 1. DMEM additives for each cell line and the number per well for the experiments.

Cell line	**DMEM additives**	**Number per well on TiO_2 coatings**	**Number per well in presence of TiO_2 and SiO_2 powders in the culture medium**
NIH-3T3	10 % NCS	3.000	6.000
HEP-G2	10 % FCS	5.000	10.000
A-549	10 % NCS	4.000	8.000
PC-12	10 % HOS, 5% NCS, 1 % sodiumpyruvate, 1 % L-glutamine	5.000	10.000

2.2. Cultivation on TiO_2 powder coatings

Following their autoclavation, the powders (Table 2) were suspended in the culture medium of the accordant cell line. The wells were coated with a suspension containing 0.1 % powder in the culture medium. 200 µL of the different TiO_2 suspensions were added to every well of a 96 well plate and

incubated for 4 days. After 4 days the medium was removed and the wells could be seeded with a defined number of cells (see Table 1). The cells were cultivated over a period of 14 days and the viability of the cells was determined by the MTT-test on day 1, day 4, day 7, day 11, and day 14.

2.3. Cultivation in TiO_2 suspensions

Wells of a 96 well plate were seeded with a defined number of cells (see Table 1). The plates were incubated 4 days (37°C / 5% CO_2) and on day 5 the particles (Table 2) were added to the cells at a concentration of 0.1% in the culture medium of the accordant cell line. The viability of the cells was determined on day 7 using the MTT-test.

2.4. Cultivation in SiO_2 suspensions

Wells of a 96 well plate were seeded with a defined number of cells (see Table 1). 200 µL of the different SiO_2 suspensions (Table 3) in a concentration of 0.1% were added to every well. The cells were cultivated over a period of 14 days and the viability of the cells was determined by the MTT-test on day 1, day 4, day 7, day 11, and day 14.

2.5. Cell metabolism

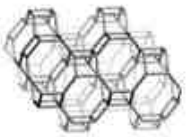
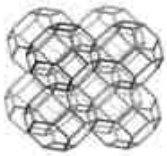
The cells were cultivated in 96-well plates. At regular intervals the viability of the cells was analysed by MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromid) test (Sigma-Aldrich, Steinheim, Germany). This assay is based on the hydrolysis of the tetrazonium ring by mitochondrial dehydrogenase enzymes to an insoluble blue reaction product.

To perform the MTT-test, at first medium of each well had to be removed. Afterwards, 100 µl of fresh medium and 10 µl of MTT solution (5 mg/ml PBS, sterile) were added to each well and incubated for 4 h at 37°C / 5% CO_2. Subsequently, 100 µl of 10% SDS in 0.01 M HCl was added and the plates were further incubated for 24 h. The transmission signals at 570/630 nm was determined using a microplate reader (Bio-Rad, München, Germany).

Table 2. Physical data (crystal structure, size and BET surface area) of the tested TiO_2 nanoparticles : PC 10, PC 50, PC 100, PC 500, UV 100, P 25, R 15, R 25, R 34.

Pulver	Kristallstruktur	Größe der Nanopartikel (nm)	BET-Oberfläche (m^2/g)
PC 10	100 % Anatas	152	10
PC 50	100 % Anatas	40	50
PC 100	100 % Anatas	26	90
PC 500	100 % Anatas	7	340
UV 100	100 % Anatas	5-13	290
P 25	80 % Anatas 20 % Rutil	37 (Anatas) 90 (Rutil)	50
R 15	100 % Rutil	20	65
R 25	100 % Rutil	27	42
R 34	100 % Rutil	36	33

Table 3. Elemental formula and structure of the tested SiO_2 nanoparticles : MFI, SOD, GOS

Particle	Elemental formular	structure
nano-Tetrapropylammonium-Silicalith-1 (TPA-MFI)	$[(C_{12}H_{28}NOH)_4[Si_{96}O_{192}]$-MFI	
nano-Tetramethylammonium-Gismondin (TMA-GIS)	$[(C_4H_{12}N)_4[Al_4Si_{12}O_{32}]$-GIS	
nano-Tetramethylammonium-Sodalith (TMA-SOD)	$[C_4H_{12}N)_2]$ $[Al_2Si_{10}O_{24}]$-SOD	

3. Results and Discussion

3.1. Cultivation on TiO_2 powder coatings and in TiO_2 suspensions

After the cells were seeded onto the different TiO_2 powders or alternatively after the cells were cultivated in presence of the powders in the culture medium, the viability was analysed using the MTT-test. Table 2 shows the different physical properties of the tested TiO_2 nanoparticles [15].

Figures 1-4 show the viability of the selected cell lines cultivated on the TiO_2 powders coatings over a period of 14 days. The NIH-3T3 cells show the lowest viability on the Aeroxide TiO_2 P 25 powder coating. On the rutile powder coatings (R 15, R 25, R 34) the NIH-3T3 cells reach a plateau and do not grow anymore (Figure 1).

As can be seen in Figure 2, the HEP-G2 cells only exhibit on the PC 10 and the PC 50 powder coatings a similar proliferation as the cells cultivated on the uncoated cell culture flask surface. No viability of the cells is observable on the R 34 powder coatings on day 7, 11 and 14. As shown in Figure 3 all A-549 cells cultivated on the powder coatings exhibit a lower viability as compared with the cells cultivated on polystyrene, with the viabilities of the cells on the rutile powders being lower than the viabilities on the anatase powders. PC-12 cells cultivated on the powder coatings as compared with all show a lower viability like the cells cultivated on PLL (Figure 4), but a higher viability than the cells cultivated on polystyrene, except for R 34.

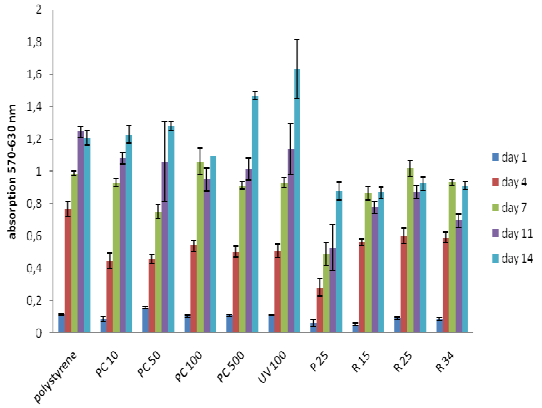


Figure 1. Viability of the NIH-3T3 cells cultivated on the different TiO_2 powder coatings over a time period of 14 days ± the standard deviation.

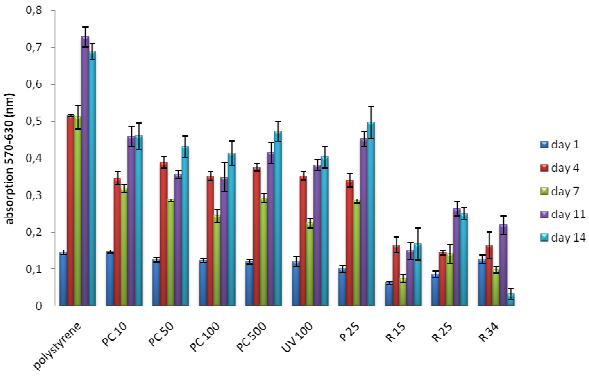


Figure 3. Viability of A-549 cells cultivated on the different TiO_2 powder coatings over a time period of 14 days ± the standard deviation.

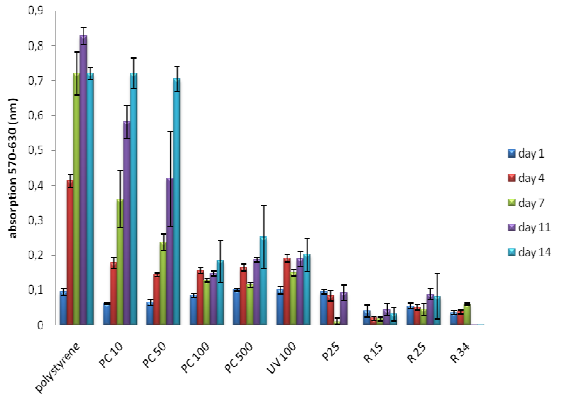


Figure 2. Viability of the HEP-G2 cells cultivated on the different TiO_2 powder coatings over a time period of 14 days ± the standard deviation.

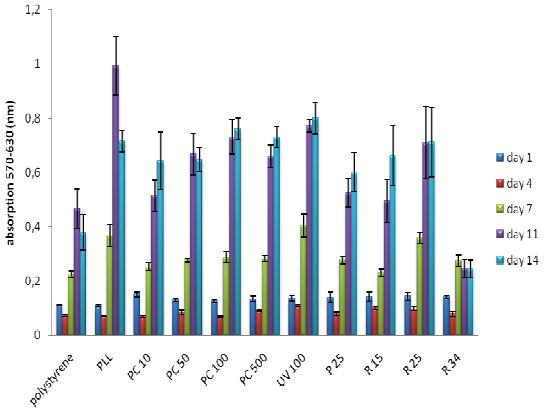


Figure 4. Viability of PC-12 cells cultivated on the different TiO_2 powder coatings over a time period of 14 days ± the standard deviation.

Figures 5-8 show the cell viability data for the cultivations carried out in the presence of the TiO_2 powders at a concentration of 0.1% in the culture medium of the according cell line. It is obvious from the results shown in these figures that the addition of the titanium dioxide powders at a concentration of 0.1% to the cell culture medium has no effect on the growth of the NIH-3T3 cells (Figure 5). The cell growth of the HEP-G2 cells in presence of the powders at a concentration of 0.1% in the culture medium was only effected by the R 34 powder (Figure 6). As shown in Figure 7 all powders at a concentration of 0.1% to the cell culture medium have an inhibitory effect on the viability of the A-549 cells. Only PC 100 and P 25 at a concentration of 0.1% have a negative effect on the viability of the PC-12 cells (Figure 8).

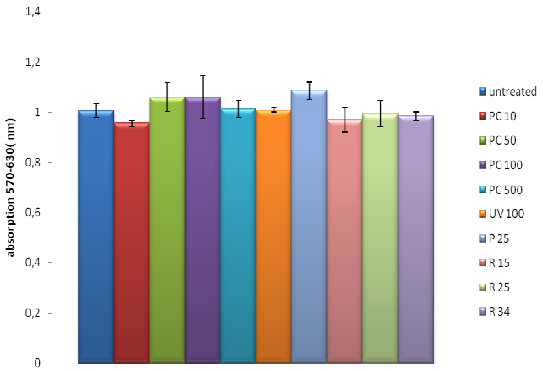


Figure 5. Viability of NIH-3T3 cells cultivated with powder presence of 0.1% in the cell culture medium ± SD.

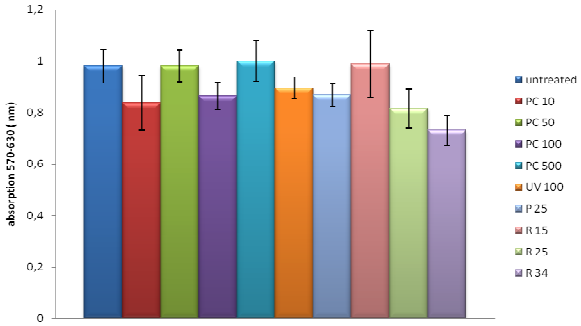


Figure 6. Viability of HEP-G2 cells cultivated with powder presence of 0.1% in the cell culture medium ± SD.

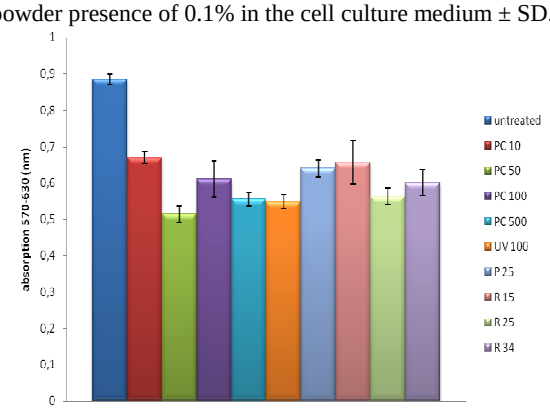


Figure 7. Viability of A-549 cells cultivated with powder presence of 0.1% in the cell culture medium ± SD.

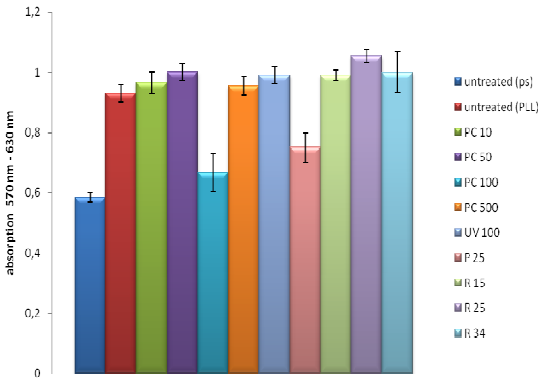


Figure 8. Viability of PC-12 cells cultivated with powder presence of 0.1% in the cell culture medium ± SD.

3.2. Cultivation in SiO_2 suspensions

Figures 9-12 show the cell viability data for the cultivations carried out in the presence of the SiO_2 powders at a concentration of 0.1% in the culture medium of the according cell line over a period of 14 days.

As can be seen in Figure 9 all HEP-G2 cells cultivated in presence of 0.1% of the SiO_2 particles exhibit a lower viability as compared with the cells cultivated in DMEM without particles. The addition of 0.1% of the SiO_2 particles to the cell culture exhibit no toxic effect to the NIH-3T3 cells (Figure 10), to the PC-12 cells (Figure 11) and to the A-549 cells (Figure 12).

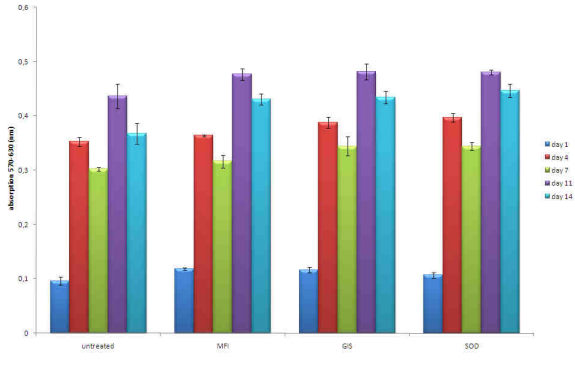


Figure 9. Viability of HEP-G2 cells cultivated in presence of SiO_2 in the culture medium over a time period of 14 days ± the standard deviation.

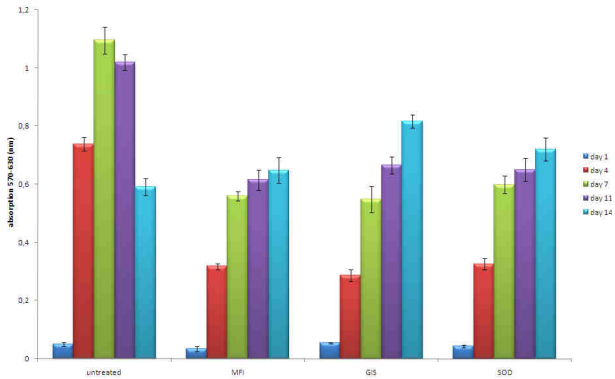


Figure 10. Viability of NIH-3T3 cells cultivated in presence of SiO_2 in the culture medium over a time period of 14 days ± the standard deviation.

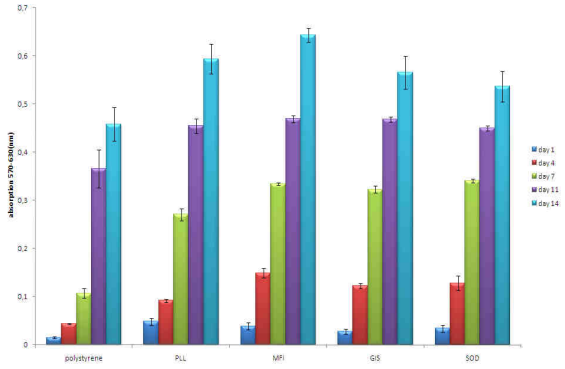


Figure 11. Viability of PC-12 cells cultivated in presence of SiO_2 in the culture medium over a time period of 14 days ± the standard deviation.

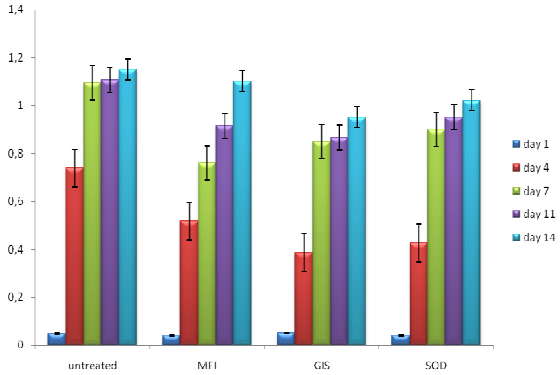


Figure 12. Viability of A-549 cells cultivated in presence of SiO_2 in the culture medium over a time period of 14 days ± the standard deviation.

4. Conclusions

Nanoparticles are less than 100 nm in size and possibly pose a health risk to humans. If they have been incorporated into the human body they can move freely because their size is similar to that of typical cellular compounds and proteins. Titanium Dioxide is because of its photocatalytic activity multifunctional applicable. Applications of TiO_2 nanoparticles are for example antibacterial and self cleaning surfaces and the treatment of wastewater and the air. Nanoparticles change their physical and chemical properties in respect to solid states, but about the change of the biological properties is so far little known. For this reason the biological harmlessness of these particles is importantly.

The objectives of this study were to assess the cytotoxic responses of four different mammalian cell lines exposed to nine unequal TiO_2 nanoparticles, which differ in their size, their BET surface area and their crystal structure and to 3 unequal SiO_2 nanoparticles. With regard to the possible absorption of nanoparticles into the human organism: via skin, via respiratory tract and via alimentary system, the toxicity was tested on connective tissue (NIH-3T3), liver (HEP-G2), lung (A-549) and kidney (PC-12).

For testing the effect of the nanoparticles on the different cells, the cells were cultivated on the powder coatings and in cell culture medium containing powders. The cytotoxic effect of the powders was determined via MTT test, a well established in vitro assay.

The experimental results have shown that the adhesion of the cells cultivated on the different TiO_2 powder coatings highly depends on the type of the cells on the type of powders. To exclude that the lower viabilities of the cells are not caused by problems in the adhesion of the cells on the TiO_2 powder coating surfaces, but due to a possible cytotoxic effect of the powders, the cells were cultivated in presence of the Titanium dioxide powders in the culture medium. These results show that the TiO_2 nanoparticles have no toxic effect on the cells. All cells are still dividable. Furthermore it could be shown that the physical properties of the particles do not refer to any observed biological effect. The tested SiO_2 nanoparticles also exhibit no toxic effect on the cells.

The concentrations of the particles tested in this study are relatively high. The TiO_2 nanoparticles concentrations in cosmetics or on self-cleaning surfaces for example are less. Consequently, he tested particles pose no danger for humans in the investigated concentration area. To get more detailed information about the possible cytotoxic effect of nanomaterials to mammalian cell lines more researches are necessary. Of great interests are researches which include the mechanism of interaction between the cells and the nanoparticles. Also important is to find out in which area of the cells when the particles are incorporated they are located.

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