University of Pardubice

Faculty of Chemical Technology

Assessment of cellular effects of nanomaterials

Doctoral Thesis

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Mgr. Jana Báčová

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Abstract

Nanomaterials have been commonly used for various purposes based on their unique properties. However, nanomaterials can enter organisms due to their small size and induce biological effects in cells. In the last decade, attention has been given to studies investigating the biological effects of nanomaterials, i.e., biocompatibility or cytotoxicity. The present PhD thesis has been focused on study of biological effects of nanomaterials *in vitro*. Initially, we optimized the experimental conditions of TiO_2 P25 nanoparticles preparation for testing in human pulmonary A549 cells. When the optimal parameters for the use of TiO_2 P25 nanoparticles in A549 cells were defined, we were able to obtain reproducible results showing mild cytotoxicity of TiO_2 P25 nanoparticles.

We also estimated the biological effects of Al_2O_3, SiO_2, ZrO_2, TiO_2, and WO_3 nanoparticles in comparison to fibers of the same chemical composition. In general, our comprehensive study provided findings that inorganic fibers did not exhibit larger toxicity in A549 cells in comparison to appropriate nanoparticles.

In addition, we focused on investigation of the biological effects of TiO_2-based nanomaterials including aerogel TiO_2 nanopowder, TiO_2-based adsorbents, and sheets and nanotubes modified by Atomic Layer Deposition technique. These nanomaterials did not cause any significant damage of human cells in contrast to multi-walled carbon nanotubes. The surface modification by Atomic Layer Deposition significantly increased the biocompatibility of materials. In conclusion, the results in the present PhD thesis provide a comprehensive view on biological effects of nanomaterials in cultured cells.

Keywords: titanium dioxide; nanoparticles; fibers; A549 cells; Atomic Layer Deposition.

Abstrakt

Nanomateriály jsou běžně využívány pro různé účely díky jejich jedinečným vlastnostem. Nanomateriály kvůli své malé velikosti mohou vstupovat do organismů a vyvolávat tak biologické účinky v buňkách. V posledním desetiletí je věnována pozornost studiím zkoumající biologické účinky, tedy biokompatibilitu či cytotoxicitu nanomateriálů. Současná Ph.D. práce je zaměřena na studium biologických účinků nanomateriálů *in vitro.* Nejprve jsme optimalizovali experimentální podmínky přípravy nanočástic TiO_2 P25 pro testování v lidských plicních buňkách A549. Po nalezení optimálních parametrů pro testování nanočástic TiO_2 P25 na buňkách A549 jsme získali reprodukovatelné výsledky prokazující mírnou cytotoxicitu nanočástic TiO_2 P25.

Stanovili jsme také biologické účinky vláken Al_2O_3, SiO_2, ZrO_2, TiO_2 a WO_3 v porovnání s nanočásticemi stejného chemického složení. Obecně z naší komplexní studie vyplývá, že anorganická vlákna neindukovala větší toxicitu v buňkách A549 ve srovnání s vhodnými nanočásticemi.

Poté jsme se zaměřili na zkoumání biologických účinků nanomateriálů na bázi TiO_2 včetně TiO_2 nanočástic s modifikovanými vlastnostmi, adsorbentů na bázi TiO_2, fólií a nanotrubic modifikovaných technikou depozice atomárních vrstev. Tyto nanomateriály nezpůsobily žádné významné poškození lidských buněk na rozdíl od vícestěnných uhlíkových nanotrubic. Navíc úprava povrchu pomocí depozice atomárních vrstev výrazně zvýšila biokompatibilitu materiálů. Závěrem lze shrnout, že výsledky uvedené v této Ph.D. práci poskytují komplexní pohled na biologické účinky nanomateriálů v kultivovaných buňkách.

Klíčová slova: oxid titaničitý; nanočástice; vlákna; buňky A549; depozice atomárních vrstev.

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List of abbreviations

ALD	Atomic Layer Deposition
CM-H2DCFDA	Chloromethyl-2',7'-dichlorodihydrofluorescein diacetate
DLS	Dynamic light scattering
FBS	Fetal bovine serum
GSH	Glutathione
MCB	Monochlorobimane
MWCNTs	Multi-walled carbon nanotubes
NMs	Nanomaterials
NPs	Nanoparticles
ROS	Reactive oxygen species
SEM	Scanning electron microscope
TEM	Transmission electron microscopy
TiAP	TiO_2 aerogel powder
TNT	Titanium dioxide nanotubes
WST-1	Tetrazolium salt (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5 -tetrazolio]-1,3-benzene disulfonate)

Introduction

Currently, a variety of nanomaterials with specific physical and chemical properties are being developed worldwide. With the increasing applications of these nanomaterials, the question of their safety has been raised. Understanding the consequences of the interaction of nanomaterials with cells is a key prerequisite of a subsequent use in any biological applications.

All the experiments concerning nanomaterials were performed as a part of EDRF NANOBIO project (No. CZ.02.1.01/0.0/0.0/17_048/0007421). Their aims ranged from optimization of experimental conditions for biological testing of nanomaterials to evaluation of safety of nanomaterials (i.e., nanoparticles, fibers, nanotubes), especially in the pulmonary A549 cell line. The most of tested nanomaterials were developed at the Center of Materials and Nanotechnologies (CEMNAT), Faculty of Chemical Technology, University of Pardubice. Finally, I, as an author, suppose that the studies on testing the biological effects of newly synthesized nanomaterials provide primary findings that can serve as a basis for following scientific studies.

The present PhD thesis is a compilation of five topical reports evaluating the cellular effects of newly developed and commercially available nanomaterials. The reports are numbered as #1, #2, #3, #4, and #5 throughout the text. Their detailed overview has been noted on page 34 followed by the full texts of the reports.

1. Theory

1.1 Nanomaterials

Nanomaterials (NMs) include a broad spectrum of materials consisting of inorganic, organic, and hybrid structures. A nanomaterial refers to a material with at least one dimension in the range of 1 to 100 nm (by definition of ISO/TS 80004 ≤ 100 nm) (Gleiter 2000, Grimsdale and Müllen 2005). Nanotechnology and nanoscience are rapidly developing fields of study in the 21st century and the commercial use of NMs for novel applications is increasing exponentially (Liu et al. 2017b). The difference between nanoscience and nanotechnology is that nanoscience gives knowledge about the structure and composition of atoms and their properties at the nanoscale and is essential for future developments. On the other hand, nanotechnology is a technology used for synthesis and preparation of new NMs (Adams and Barbante 2013).

The synthesis of NMs can be carried out in two main ways such as bottom-up and top-down approaches (Fig. 1). The bottom-up or constructive method is the synthesis of a material from atoms to clusters forming nanoparticles which includes sol-gel method, chemical vapor deposition, pyrolysis, or biosynthesis (Baig, Kammakakam and Falath 2021). In top-down approaches, bulk materials are divided or milled to produce nanostructured materials. Top-down methods include especially mechanical milling, laser ablation, etching, sputtering, electro-explosion, or electrospinning or centrifugal spinning (Wang et al. 2020). The centrifugal spinning is a very modern and industrially robust technique for production of fibers. Centrifugal spun fibers are formed due to high centrifugal force applied on the solution in the rotating spinneret, which has numerous nozzles with diameter of μm. When the equilibrium between the surface tension and the centrifugal force is disrupted, the solution is expelled from the nozzles of the spinneret in the form of many jets of fibers (Rihova et al. 2021). The advantage of centrifugal spinning method is quantitative production of fibers with same or similar diameters.

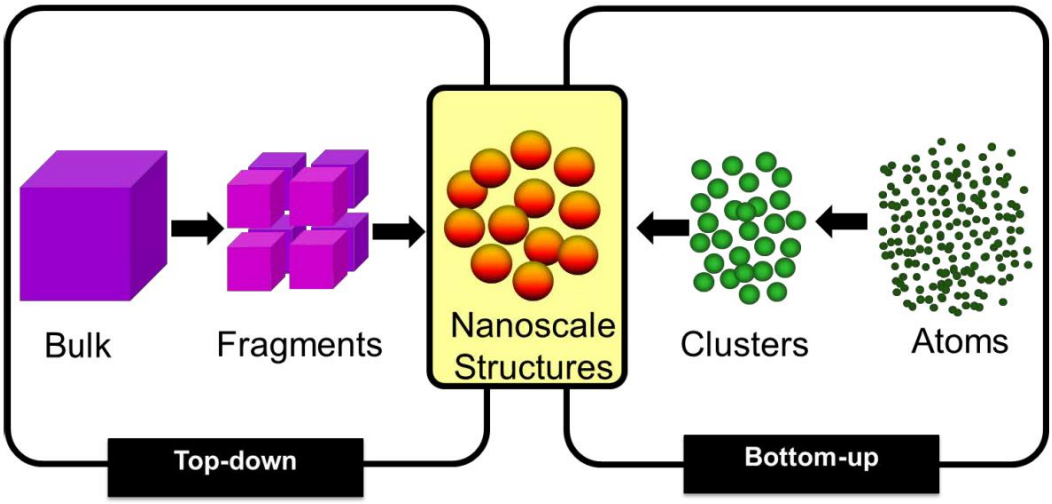

Fig. 1: "Top-down" and "bottom-up" synthesis of nanomaterials (Adapted from Rawat 2015).

1.1.1 Classification of nanomaterials

The nanomaterials differ in dimension, shape, size, and material. Based on nanoscale dimensions, they are classified into four categories (Fig. 2) including zero-dimensional, one-dimensional, two-dimensional, and three-dimensional NMs (Baig et al. 2021).

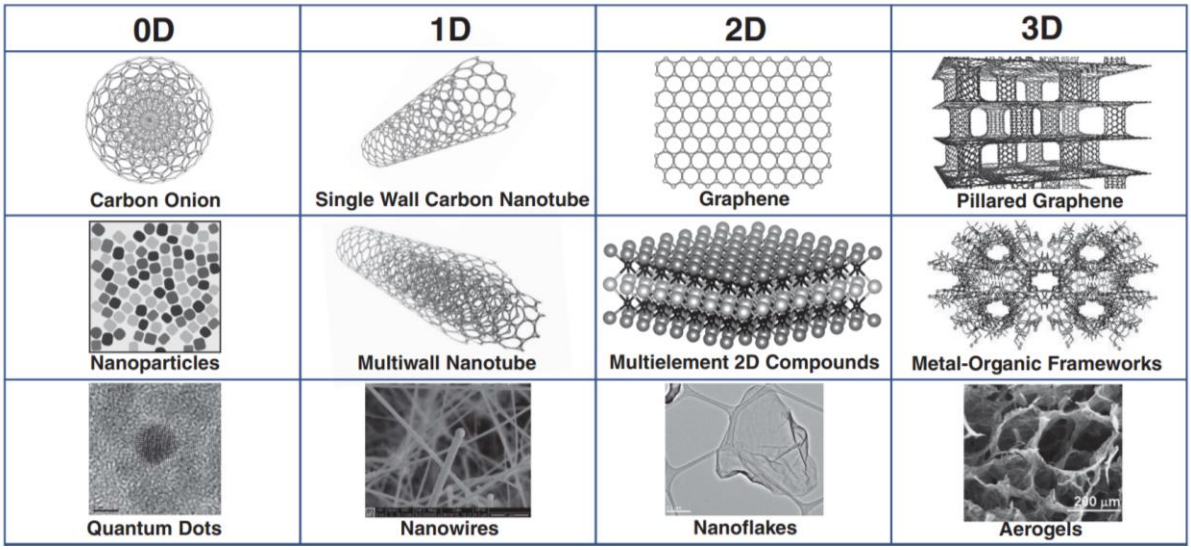

Fig. 2: Classification of nanomaterials (Adapted from Pomerantseva et al. 2019).

Zero-dimensional nanomaterials (0D) have a length, breadth, and height fixed at a single point, for example, quantum dots, fullerenes, or nanoparticles (NPs). The considerable selectivity and reactivity of NPs caused by their very small size have produced a wide range of applications. Nanoparticles of different chemical composition are shown in Table 1. TiO_2 NPs, especially TiO_2 P25 NPs, are one of the most widely used nanomaterials in the products (Allegri et al. 2016, Michalkova et al. 2020).

Tab. 1: Metal oxide-based nanoparticles and their applications.

Nanoparticles	Applications
Al_2O_3	Catalyst, pharmaceutical and material manufacturing industries
CeO_2	Catalytic activity, corrosion protection, solar cells
Fe_2O_3	Magnetic storage devices, magnetic resonance imaging, catalysis
SiO_2	Delivery of drugs and other biomolecules, glass products
TiO_2	Photocatalysts, creams and coatings, disinfection of wastewater
WO_3	Catalysis, photoelectrode, electrochromic devices
ZnO	Semiconductors, sunscreen, white pigment
ZrO_2	Ceramic pigments, abrasive, and fire-retarding materials

In the case of one-dimensional nanomaterials (1D), one dimension is outside the nanoscale range and the remaining two dimensions are in the nanoscale range. Thus, nanotubes, nanofibers, or nanowires belong to this group.

Two-dimensional nanomaterials (2D) are nanomaterials with sheet-like structures and transverse dimensions larger than 100 nm, while the thickness is typically less than 5 nm. This group includes graphene, nanofilms, nanolayers, and nanosheets.

Three-dimensional or bulk nanomaterials (3D) are materials that are not confined to the nanoscale in any dimension. This class can contain bulk powders, bundles of nanowires, nanocomposites, core shells, polycrystals, organic metal frameworks as well as multi-nanolayers.

1.1.2 Physicochemical properties

The nanomaterials exhibit unique chemical and physical properties due to nanoscale size compared to their respective materials of larger scales. This feature is based on relatively larger surface area than the volume (Karakoti, Hench and Seal 2006). The number of surface molecules increases exponentially when the particle decreases in diameter. Therefore, nanoparticles have a larger percentage of surface molecules and atoms compared to the same materials of larger dimension (Oberdörster, Oberdörster and Oberdörster 2005). The chemical properties of NMs such as reactivity, heat/light/mechanical stability, or capability to moisture the atmosphere determine their future applications (Ealia and Saravanakumar 2017).

NMs also differ in physical properties such as optical, mechanical, magnetic, and electrical properties. Hydrophilicity, hydrophobicity, suspension, diffusion, settling characteristics and optical and mechanical properties play an important role in the use of NMs in many modern applications. Magnetic and electrical properties of NPs such as conductivity and resistivity have led to the use of NPs in transistors, electrical switching, nanoelectronics, sensors, or electronics in renewable energy applications (Zhang et al. 2020, Nazir et al. 2021).

As shown in recent reports, nanotechnology can dramatically change the properties and applications of NMs (Bayda et al. 2019, Adams and Barbante 2013). Nanoparticles are composed of primary and agglomerated particles that can vary in size, shape, charge, crystallinity, chemical composition, and other characteristics, and this variety will increase further (Gea et al. 2019, Prasad et al. 2013). TiO_2 NPs occur in three crystalline structures, including rutile, brookite or anatase (Fig. 3).

It is known that TiO_2 P25 NPs are composed of anatase and rutile crystallites, the reported ratio being typically 80:20. Anatase, rutile, or anatase/rutile mixtures occur naturally and have been used in various applications including industrial, electrical, pharmaceutical and biomedical fields (Zarhri et al. 2020, Tian et al. 2015). Anatase and rutile forms have been used in photocatalytic studies (Wold 1993, Bertolotti et al. 2020). Anatase appears to be more active compared to rutile due to differences in the extent and nature of surface hydroxyl groups present in the structure of anatase (Carp, Huisman and Reller 2004).

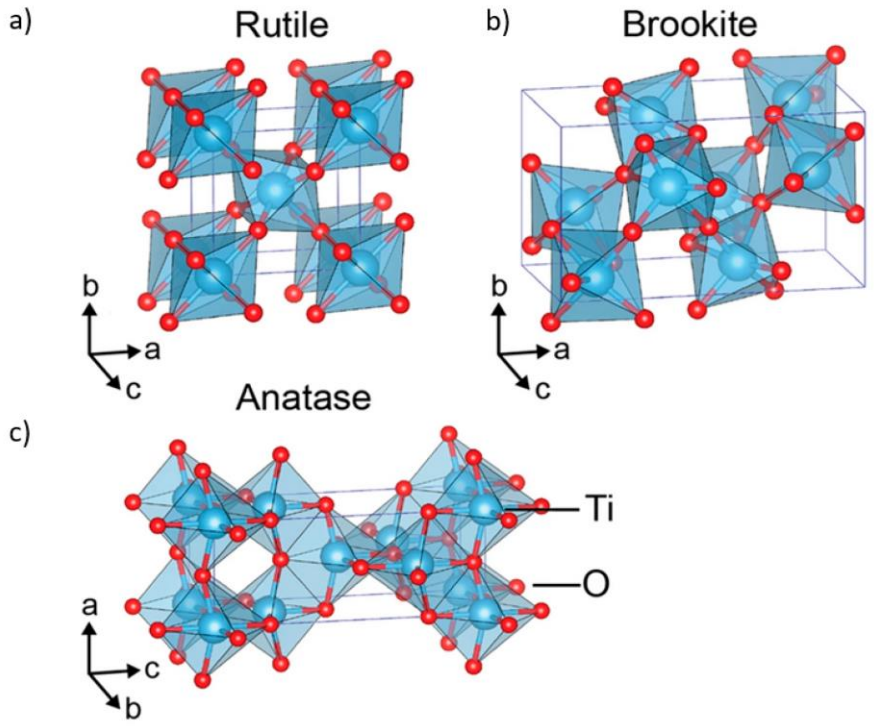

Fig. 3: Crystal structures of TiO_2 nanoparticles: rutile, brookite and anatase (Adapted from Benčina et al. 2020).

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1.1.3 Characterization of nanomaterials

Physical and chemical characterization of manufactured nanomaterials using appropriate methods is crucial to determine their properties (Ealia and Saravanakumar 2017). A wide spectrum of methods is focused on the assessment of hydrodynamic size, shape, surface area, chemical composition, crystallinity, morphology, solubility, surface charge, and dispersion stability of NMs. The various types of TiO_2 NMs obtained by scanning electron microscope (SEM) are shown in Figure 4.

Characterization of nanomaterials is essential to estimate the homogeneity, stability, reactivity, and potential application of NMs in different environments (Moore et al. 2015). A short overview of analytical techniques being applied to characterization of NMs is summarized in Table 2. Moreover, average size and elemental composition, which can be related to their behavior, should always be determined in reports (Stone et al. 2010, Peralta-Videa et al. 2011).

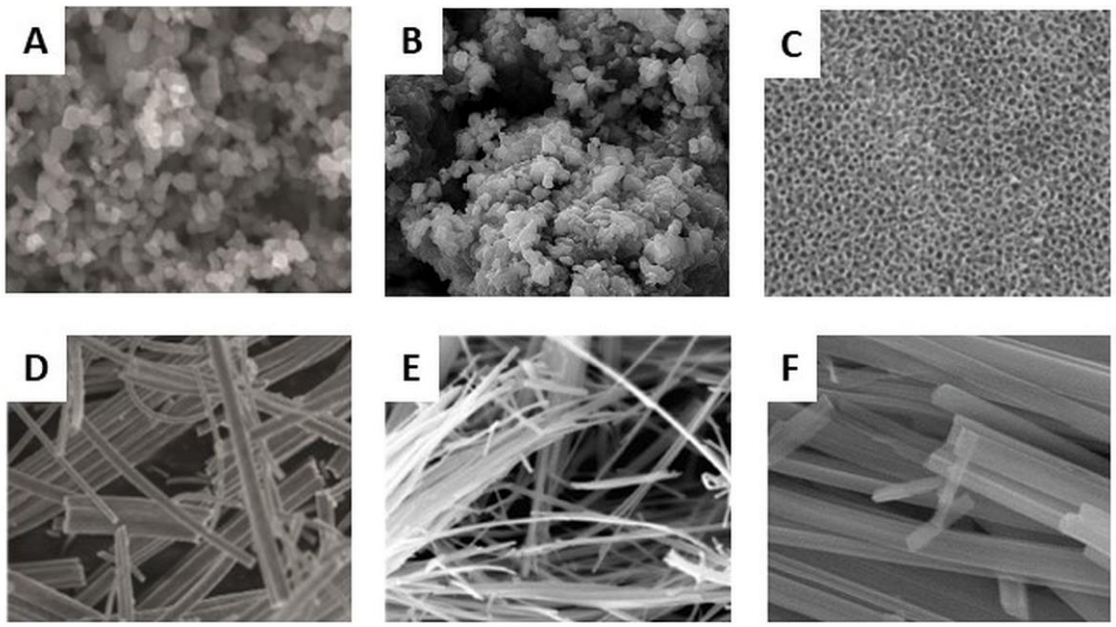

Fig. 4: Photomicrographs of TiO_2 materials: (A) TiO_2 P25 nanoparticles (Adapted from Report #1)**, (B) TiO_2-based adsorbent** (Adapted from Report #4)**, (C) TiO_2 nanotubes** (Adapted from Report #5), **(D) TiO_2 fibers** (Adapted from Report #2), **(E) TiO_2 nanowires** (Adapted from Zhang et al. 2002) **and (F) TiO_2 nanobelts** (Adapted from Li et al. 2013); characterized using scanning electron microscopy.

The size of observed clusters of nanoparticles is often affected by their ability to form agglomerates or aggregates in different environments (Pellegrino et al. 2017). An agglomerate is formed by particles into a ball or cluster that is merged into a mass. Agglomerates are assemblages of particles rigidly joined together, as by partial fusion (sintering) or by growing together that are not readily dispersed. On the other hand, an aggregate is formed by the union of individual particles. Aggregates are assemblages of particles that are separable from each other. Unfortunately, these terms are frequently interchanged with conflicting definitions in international standards, and this can result in universal confusion of outcomes of the scientific reports (Nichols et al. 2002). It was found that various TiO_2 NPs obtained from different commercial sources formed agglomerates. These agglomerates were not further separable using sonication (Jayaram and Payne 2020, Jiménez-Chávez et al. 2021). For example, we determined the primary size of TiO_2 P25 NPs (Sigma-Aldrich, USA) 30±10 nm, but TiO_2 P25 NPs are commercially supplied in the form of agglomerates with an average size greater than 1 μm in maximal dimension. Moreover, characterization of physicochemical properties of nanomaterials is important for further assessment of behavior of nanomaterials *in vitro.*

Tab. 2: Overview of characterization methods used for nanomaterials.

Method	Abbr.	Analyzed parameter
Atomic force microscopy	AFM	Size, size distribution morphology, surface texture and roughness, agglomeration, aspect ratio
Capillary electrophoresis	CE	Size
Dynamic light scattering	DLS	Size, size distribution, surface charge, agglomeration
Energy-dispersive X-ray spectroscopy	EDX	Elemental analysis, chemical characterization
Inductively coupled plasma mass spectrometry	ICP-MS	Elemental composition
Raman spectroscopy	RS	Crystal structure
Scanning electron microscopy	SEM	Size, size distribution, shape, agglomeration, aspect ratio, elemental composition (when combined with energy-dispersive X-ray spectroscopy)
Transmission electron microscopy	TEM	Size, shape, homogeneity, and lattice structures at atomic resolution
X-ray diffraction	XRD	Crystallography

1.2 Cellular effects of nanomaterials

Alveolar epithelial cells and alveolar macrophages (Fig. 5) represent a major target of NMs in the lungs (Stone et al. 2017). After inhalation, TiO_2 NPs can be transported through airways, alveoli and capillaries to other tissues, i.e. to the heart, liver, or brain (Wu and Tang 2018, Bhattacharya et al. 2017). Short- or long-term clearance of inhaled TiO_2 NPs from human tissues depend on the duration of inhalation and the concentration of NPs (Christensen et al. 2011). Moreover, solubility of nanoparticles affects their persistence and clearance too. Exposure to highly persistent NPs of low toxicity may result in larger pulmonary impairment and more pronounced effects than exposure to very toxic NMs with low persistence in human body (Landsiedel et al. 2014). Therefore, there is a high demand to test their pulmonary toxicity.

Nanoparticles in pulmonary cells can increase the production of reactive oxygen species (ROS) to cause severe oxidative stress *in vitro*. ROS can be generated after internalization of NPs into mitochondria or after depletion of antioxidants in cells. During exposure to NPs, enhanced ROS production is capable of inducing DNA strand

breaks, protein oxidation, lipid peroxidation, and mutagenesis (Biola-Clier et al. 2017). In addition, they can cause mitochondrial membrane damage, necrosis, and apoptosis (Wang et al. 2015). In general, oxidative stress-mediated stimulation of these cellular mechanisms leads to several pathological processes including inflammation, fibrosis, hypertrophy, metaplasia, genotoxicity, or carcinogenesis (Lewinski, Colvin and Drezek 2008).

Several cell models can be used to test the biological effects of nanomaterials. Carcinoma and immortalized cell lines often replace primary cells in research. Cell lines offer several advantages, such as being more cost effective, easy to use, providing an unlimited supply of biological material, and excluding the ethical concerns associated with the use of animal or human tissues. Cell lines also produce pure population of cells and thus can provide reproducible results (Ekstrand-Hammarström et al. 2012).

The pulmonary human A549 cell line is one of the most widely used models for testing pulmonary toxicity *in vitro* (Hufnagel et al. 2021, Wu et al. 2019). The A549 adenocarcinoma cell line was established in 1972 (Lieber et al. 1976). A549 cells provide characteristic features of pulmonary cells type II with typical lamellar bodies and consistent metabolic and transport properties. Conventional cytotoxicity testing *in vitro* compared to *in vivo* experiments using animals is less expensive, faster, and avoids ethical problems. On the other hand, *in vitro* cytotoxicity testing provides lower predictive value (Frohlich 2018). A549 cells have been widely used in reports estimating pulmonary toxicity of NMs, i.e., silica, iron oxides, zinc oxide, titanium dioxide, silver nanoparticles and other specifically functionalized nanoparticles, nanofibers, nanosheets and carbon-based nanomaterials.

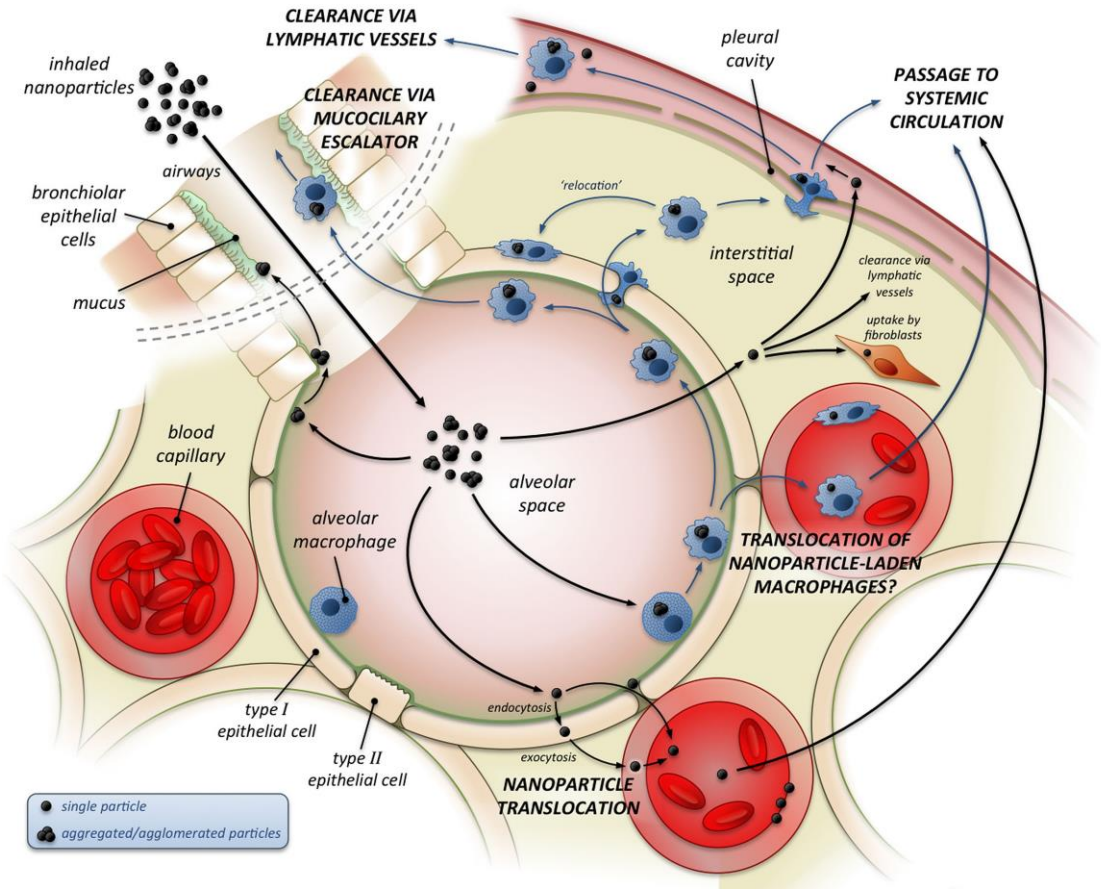

Fig. 5: Nanoparticles in lungs; inhalation and transport of nanoparticles into the epithelium and interstitial spaces, and long-term retention (Stone et al. 2017).

1.2.1 Limitations of testing of nanomaterials *in vitro*

It is important to realize that assessment of biological effects of nanomaterials can be associated with some limitations and influenced by several factors. Firstly, the preparation of nanoparticles and the preanalytical phase before their addition to the cells is important because nanoparticles in cell culture media or biological fluids containing electrolytes, proteins, and lipids can aggregate. This aspect can play a crucial role in the assessment of NPs toxicity *in vitro* or *in vivo* (Mbanga, Cukrowska and Gulumian 2022). The aggregation behavior and surface charge variation of nanoparticle dispersion can affect the reactivity of NMs (Ji et al. 2010). Another limit is a possible contamination of nanomaterials during production and handling of NMs, which can affect the assessment of biological effects *in vitro*. No less important is the

use of specific and sensitive bioanalytical assays to evaluate the effect of NMs in cells (Kose et al. 2020).

1.2.1.1 Dispersion and colloidal stability of nanomaterials

The most widely used dispersion techniques of NMs include the use of ultrasonic baths and ultrasonic probes. The inconsistent results of ultrasonic-based techniques of nanoparticle dispersion in liquid systems are caused by the variability in experimental conditions such as power, duration of the ultrasonic pulse, duration of the sonication, etc. (Tedja et al. 2011, Remzova et al. 2019). These aspects can lead to significant variability in suspension characteristics across studies.

In Report #1, based on different dispersion conditions in other studies, we compared the use of four different methods to disperse TiO_2 P25 NPs in distilled water, i.e., manual shaking, ultrasonic probe, ultrasonic bath, and Ultra-turrax®. Our results showed that the maximal dispersion of TiO_2 P25 NPs in distilled water was obtained using ultrasonic probe or bath for at least 10 min.

Further, we investigated the effect of dispersion techniques on dispersion of nanoparticles in different environments, including water, 0.9% NaCl solution and culture medium with 10% fetal bovine serum (FBS)**.** Culture media containing 10% FBS with other important supplements are commonly used for culturing cell lines *in vitro*. We found that the presence of FBS and the use of an ultrasonic bath with defined conditions for at least 10 min led to a sufficient dispersion with the required stability of the TiO_2 nanoparticles colloid.

The formation of the protein corona on the surface of colloidal nanoparticles, i.e., protein adsorption layer (Fig. 6), can lead to toxicity reduction, enhanced circulation time, and a targeted effect of NPs. This can potentially lead to applications in pharmaceutical sciences (Lee 2017). Nowadays, it is still difficult to understand and predict the stability of nanoparticles in complex culture media. The phenomenon of nanoparticle stability and the capability to aggregate in complex physiological fluids requires further investigation (Moore et al. 2015, Tedja et al. 2012).

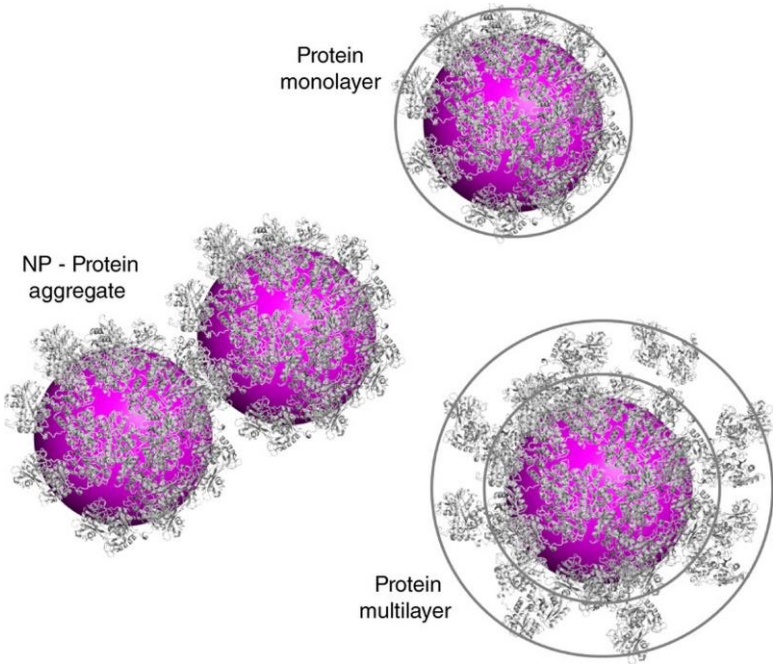

Fig. 6: Formation of protein corona on surface of nanoparticles (Wang et al. 2018).

In Report #1, we used transmission electron microscopic to assess the effect of FBS after TiO_2 P25 treatment of cells**.** We found that TiO_2 P25 NPs (anatase/rutile) were internalized into A549 cells, they occurred predominantly in cytoplasm in simple or double membrane vesicles in both the presence and the absence of FBS after 24 h of treatment. The most obvious difference between A549 cells incubated with TiO_2 P25 NPs w/wo FBS was that the nanoparticles dispersed in FBS containing medium aggregated and accumulated less around the cells in comparison to cells incubated without FBS. We concluded that the presence of FBS in cell culture medium ensures proper TiO_2 P25 NPs dispersion necessary for valuable estimation of TiO_2 P25 NPs effect in cells. Thus, we used NMs treatment of A549 cells in presence of 10% FBS culture medium in all experiments with a wide spectrum of NMs.

1.2.1.2 Reference material and endotoxin contamination

In general, biological effects of new nanomaterials on cells should be assessed with a comparative material, i.e., positive control. Commercially available reference material is used to assess the toxicity or biocompatibility of a wide range of NMs, from nanoparticles to nanotubes *in vitro*. Such an example are multi-walled carbon nanotubes (MWCNTs) obtained from the JRC Nanomaterials Repository (Møller et al. 2021). MWCNTs have been used as a reference positive control in several recent publications (Bianchi et al. 2020, Funahashi et al. 2015, Allegri et al. 2016). Carbon nanotubes can induce significant cell damage (Fig. 7). Specifically, MWCNTs caused a significantly decreased viability of pulmonary cells (Allegri et al. 2016) or alveolar macrophages depending on time (Sweeney et al. 2015). **In Reports #1, #2, #3 and #4,** we used 100 µg.mL^{-1} MWCNTs as positive reference material for the evaluation of biological effects of a broad spectrum of NMs.

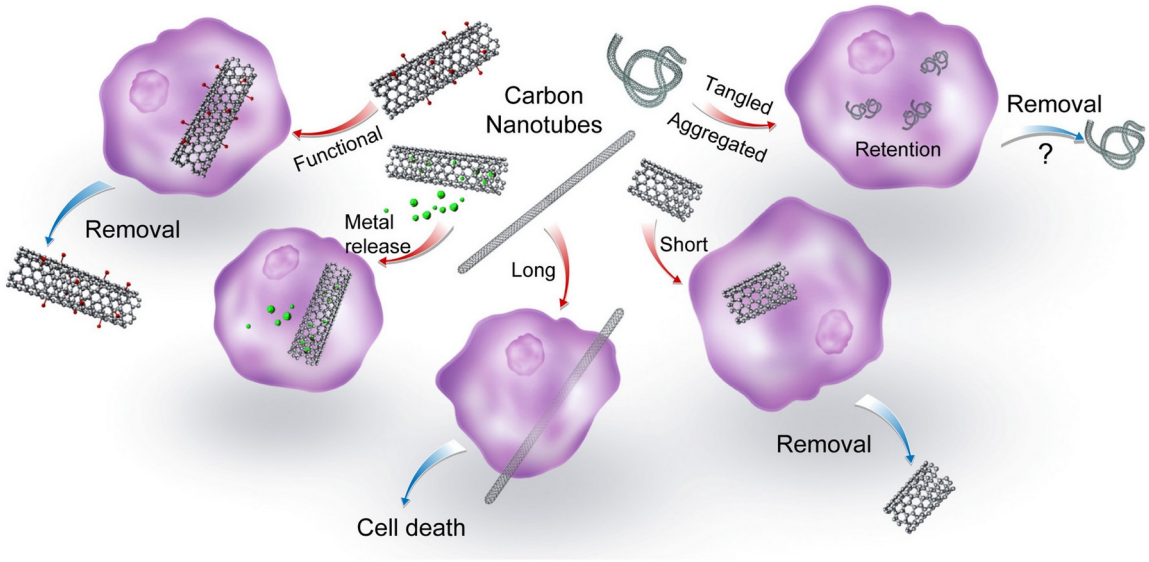

Fig. 7: The various types of carbon nanotubes and cytotoxicity effects (Liu et al. 2013).

Nanomaterials can be potentially contaminated with endotoxins. Endotoxins are lipopolysaccharides that can contaminate NMs during manufacturing, ball milling, handling, or pre-analytical preparation for biological tests (Hannon and Prina-Mello 2021). Endotoxin contamination of NMs can cause false positive results, i.e., false positive increase of inflammatory markers and reactive oxygen species (Di Cristo et al. 2016, Li and Boraschi 2016). It is essential to estimate all tested NMs for potential

endotoxin contamination. Thus, we used nanomaterials proven to be endotoxin free in all experiments and published reports.

1.2.1.3 Biochemical assays for testing biological effects of nanomaterials

The use of specific and sensitive bioanalytical assays to characterize the effect of NMs on cells plays an important role in the evaluation of safety or toxicity of NMs. In several studies a number of experimental challenges and issues were described for proper estimation of NMs biological effects (Kose et al. 2020, Jiménez-Chávez et al. 2021). Most of the methods in chemical toxicology evaluating metabolic activity of cells, oxidative stress, or genotoxicity were used for the assessment of toxicity of NMs. However, nanomaterials, and even more nanoparticles exhibit several unique physicochemical properties that can cause the interference with commonly used tests for assessment of cell toxicity (Kroll et al. 2012).

Frequently used and simple methods for toxicity assessment are based on the measurement of the metabolic activity of cultured cells by evaluating the transformation of tetrazolium salts into formazan. Formazan-based methods, including MTT, MTS, XTT, and WST assays, detect changes in spectral properties after reduction of tetrazolium salts. However, some of the assays can provide results influenced by interference of NMs with the method.

The experimental interference was reported during the testing the cellular effects of TiO_2 NPs in the case of MTT (Kroll et al. 2012, Guadagnini et al. 2015) or the neutral red assay (Guadagnini et al. 2015). Furthermore, Ag nanoparticles and Fe_2O_3 nanoparticles showed interference with MTT, MTS, and WST-8 assays (Vrček et al. 2015). The WST-1 test appears to be the most suitable for evaluating the biological effect of TiO_2 NPs. The unsuitable experimental protocol and detection principle could be also the reason for different results in published reports testing the metabolic activity of cells treated with TiO_2 NPs (Guadagnini et al. 2015, Simon-Deckers et al. 2008). Despite those reports, also recent studies have not taken into account the aspect of interference of NMs with formazan-based methods (Ong et al. 2014, Vrček et al. 2015). It is needed clearly to define and standardize experimental protocols to evaluate the biological effects of NMs. For this reason, occurrence of an interference of tested NMs with used assays should be evaluated.

Furthermore, NMs can induce production of ROS causing cellular oxidative stress. The extent of ROS generation can depend on the crystalline phase of the nanomaterials. Anatase TiO_2 NPs induced greater ROS production and cell responses than rutile TiO_2 NPs due to the active sites on surface and the different crystal lattice of anatase TiO_2 NPs (Wang and Fan 2014). Cell oxidative damage can be monitored by detection of ROS induced by NMs using a fluorescence probe chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate (CM-H2DCFDA). According to ROS production, the level of the antioxidant defense should be evaluated in the cells treated with NMs. Glutathione (GSH), as an essential intracellular antioxidant, plays an important role in reduction of reactive oxygen species (Rosales et al. 2019, Čapek and Roušar 2021). The depletion of glutathione can be detected using optimized monochlorobimane assay (Čapek et al. 2017). The new quantitative spectrofluorometric assay that detects nuclear condensation and fragmentation in intact cells can be used for the assessment of the potential genotoxicity of TiO_2 NPs (Majtnerova et al. 2021).

1.2.2 A549 cells – effects of TiO_2 nanoparticles

In the last decade, TiO_2 nanoparticles have been extensively tested concerning their possible toxicity (Gea et al. 2019, Brandão et al. 2020). Due to the small size of the NPs, they can easily penetrate cell membranes and cause cellular impartment. The entrance of NPs into cells and their biological effects are also dependent on the presence or the absence of fetal bovine serum in cell culture media and the ability to form aggregates (Jugan et al. 2012).

The results of a number of reports have indicated that the size, shape, crystalline structure and surface of TiO_2 NPs (Fig. 8) play a critical role in understanding the induced biological response and affect the level of toxicity of nanoparticles *in vitro* (Kose et al. 2020, Fresegna et al. 2021). *In vitro* studies on the pulmonary A549 cell line have been reported most frequently describing cellular effects of TiO_2 NPs, including nanoparticles consisting of crystalline forms of anatase, rutile, or anatase/rutile mixture (Ekstrand-Hammarström et al. 2012, Hufnagel et al. 2021).

Interestingly, the reports on TiO_2 P25 NPs biological effects have provided findings on different extents of NPs-induced toxicity in A549 cells. Those studies

have differed in tested concentration, incubation period, toxicity assay, or presence of fetal bovine serum during cell cultivation (Gea et al. 2019, Hanot-Roy et al. 2016, Kose et al. 2020).

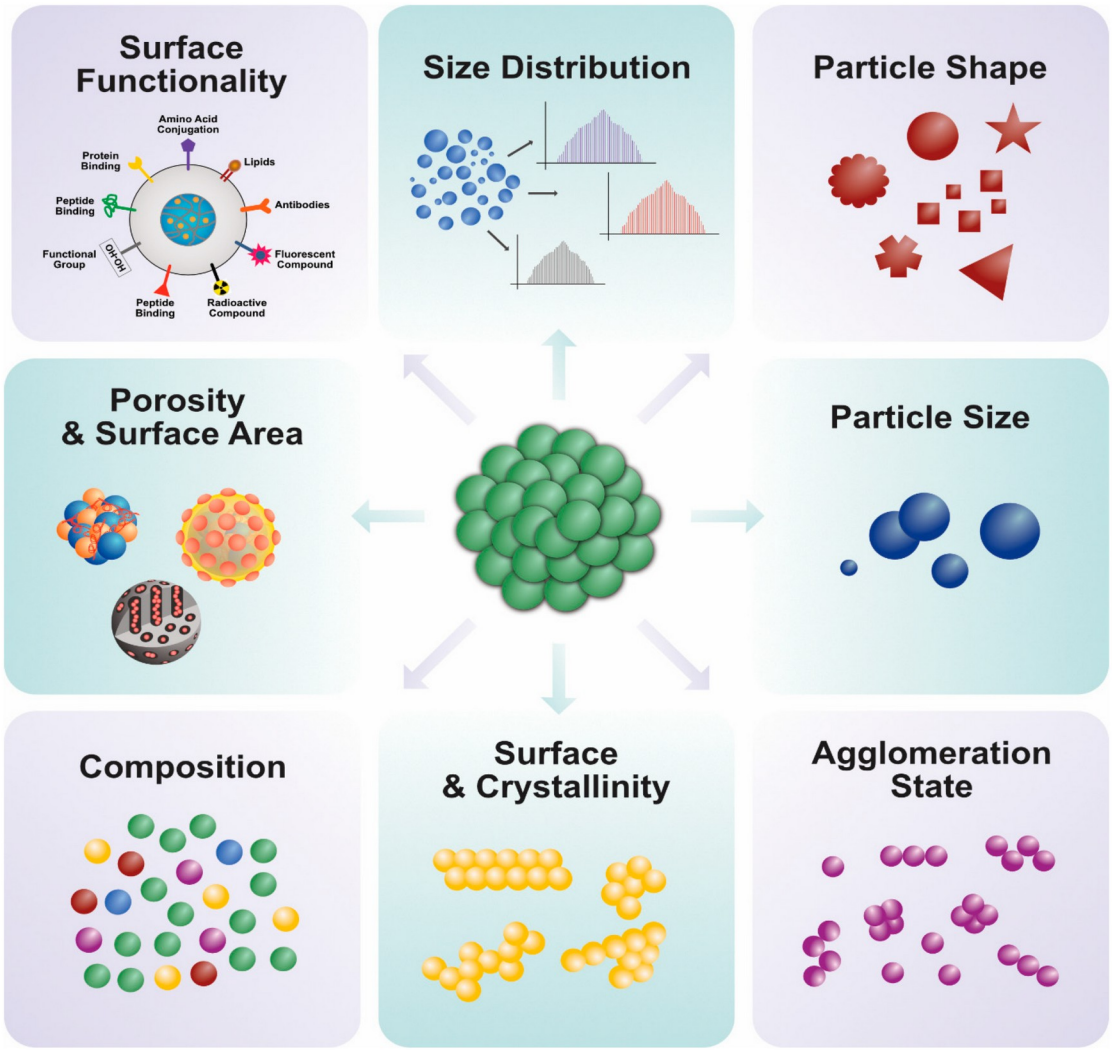

Fig. 8: Physicochemical properties of nanoparticles (Jeyaraj et al. 2019){Jeyaraj, 2019 #242;Abbass, 2018 #73}.

In our Report #1, we used A549 cells for testing of TiO_2 P25 NPs biological effects too according to the frequent use of this cellular model in the literature. After 24 h of incubation with 100 $\mu g.mL^{-1}$ TiO_2 P25 NPs, we found no significant effect on dehydrogenase activity of A549 cells. Only a slight decrease in glutathione level was observed after 24 h of exposure to 10 and 100 $\mu g.mL^{-1}$ TiO_2 P25 NPs. In addition to assessment of dehydrogenase activity and glutathione level, we used three additional methods for characterizing the TiO_2 P25 effects in A549 cells in more

detail, i.e., determination of nuclear condensation and fragmentation, ROS production and assessment of cell morphology. We did not observe any significant increase of ROS production and DNA fragmentation after incubation with TiO_2 P25 NPs compared to MWCNTs. Moreover, typical epithelial morphology was found in both untreated and TiO_2 P25-treated A549 cells.

In addition to TiO_2 P25 NPs, we tested also other NMs based on TiO_2. **In Report #3**, we compared the biological effects of TiO_2 aerogel powder synthesized by lyophilization (TiAP) and commercially available TiO_2 P25 NPs (anatase/rutile). We thoroughly compared these nanomaterials for their toxicity, photoinduced antimicrobial and antibiofilm properties, and photocatalytic activity. Regarding biological effects on A549 cells, both materials in the concentration range 1-100 $\mu g.mL^{-1}$ showed low toxicity in pulmonary A549 cells after 24 and 48 h of measurements of dehydrogenase activity and GSH level. According to the results of our study, TiAP nanopowder appears promising in environmental and biological applications mainly in term of photocatalytic efficiency.

The TiO_2-based adsorbent belongs to a special part of NMs with a large surface area, high cation exchange capacity, and high density of functional hydroxyl groups on the surface of the adsorbent (Santhosh et al. 2016, Di Giampaolo et al. 2021). Thus, **in Report #4**, we evaluated the biological effect of the newly synthesized TiO_2-based adsorbent, which can be a promising candidate for demanganization in potable water. Toxicity assessment of TiO_2-based adsorbent showed that the sorbent at the concentration of 1-100 $\mu g.mL^{-1}$ did not cause any significant damage of A549 cells after incubation up to 48 h.

We can conclude that TiO_2 NPs were not capable of inducing large cell damage compared to MWCNTs. **The Reports #1, #3 and #4** confirm that understand nanoparticle behaviors under conditions *in vitro* is very important for studies of biological effects of nanomaterials.

1.2.3 A549 cells – effects of fibers

Nanofibers belong to the group of novel NMs with a wide potential for use, i.e., in filtration applications, catalysis, health care or textile industry. By definition, nanofibers should be fibers with a diameter lower than 100 nm. However, in the publications and especially in the textile industry, fibers with as large diameter as 1 μm have been entitled as nanofibers (Rihova et al. 2021). There are essentially four classes of fibers, i.e., polymeric, metal oxide, metal, and hybrid ones. Recent technological advancements have enabled the synthesis of inorganic fibers of various chemical composition (Hromádko et al. 2017). For example, nanofibers and microfibers of amorphous SiO_2 (Fig. 9) have shown a potential as catalyst carriers or part of solid-state electrolytes of batteries (Rihova et al. 2021).

Moreover, the morphological similarity of nanofibers to pathogenic fibers such as asbestos represents a serious concern about the potential health risks after exposure of human to nanofibers (Funahashi et al. 2015). The biological effects of TiO_2 nanoparticles have been extensively studied (Brandão et al. 2020, Di Giampaolo et al. 2021). On the other hand, the toxicological characterization of TiO_2 nanofibers and other nanofibers of various chemical composition is still needed.

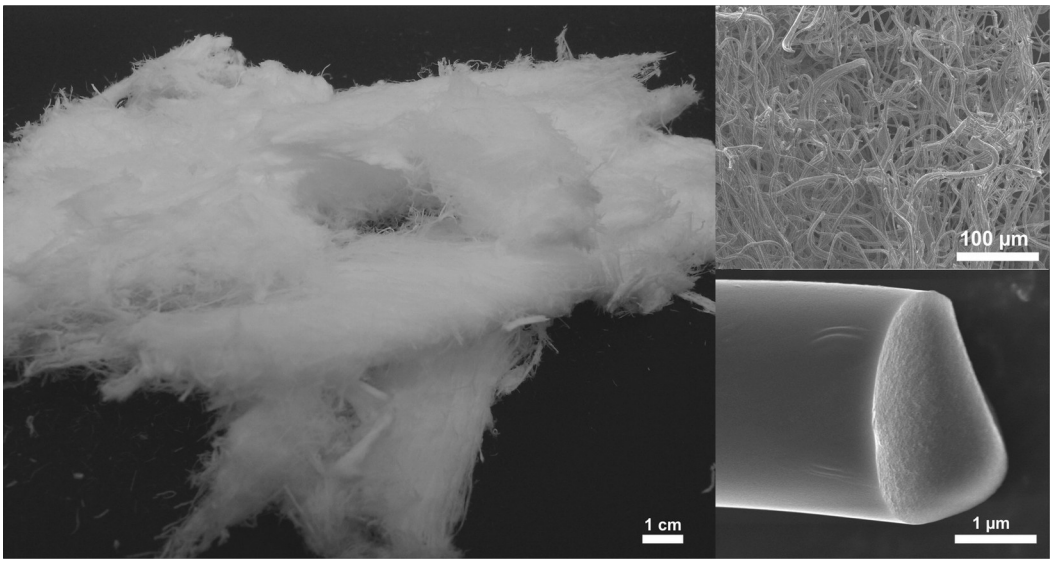

Fig. 9: SiO_2 fibers; taken using scanning electron microcopy (Hromádko et al. 2017).

In a recent study (Allegri et al. 2016), the toxicity of commercially available TiO_2 anatase nanofibers compared to TiO_2 NPs (anatase/rutile) and crocidolite asbestos as benchmark materials was evaluated on A549 cells and macrophages.

In general, nanofibers showed several toxic effects comparable to those observed with crocidolite. Furthermore, the effect of shortening of fibers was investigated on the biological effects of TiO_2 nanofibers of industrial origin using A549 cells. It is important to study the biological effect of the chemical composition of nanofibers as well as the length of the nanofibers on the cells. Long TiO_2 nanofibers were more cytotoxic compared to their shortened counterparts (Bianchi et al. 2020).

There is no study comparing the biological effects of inorganic fibers of various chemical composition entering the market. Thus, **in Report #2**, we assessed the biological effects of commercially available TiO_2, SiO_2, Al_2O_3, ZrO_2 and WO_3 fibers produced by centrifugal spinning. We estimated the potential pulmonary toxicity of newly synthesized fibers in A549 cells and compared their potential biological effects with nanoparticles of the same chemical composition. We evaluated biological effects of fibers (1-100 $\mu g.mL^{-1}$) on cell viability and GSH level in pulmonary A549 cells in comparison to nanoparticles of the same chemical composition after 24 and 48 h of exposure. We found that tested fibers exhibited no significant toxic effects in A549 cells except of Al_2O_3 and TiO_2 fibers. Al_2O_3 fibers caused significant cell damage of similar extent to Al_2O_3 nanoparticles. Similarly, we showed that TiO_2 fibers (rutile) seem to be of the same effect on cells compared to TiO_2 NPs (anatase/rutile). Generally, we found that the fibers of various chemical composition did not cause or had very mild cell impairment in pulmonary A549 cells treated only with 100 $\mu g.mL^{-1}$ concentration.

1.2.4 Atomic Layer Deposition in biomaterial surface modifications

Moreover, titanium-based nanomaterials apart from industries in many fields have been used in medicine, for example, in implantology (Kligman et al. 2021, Nazarov et al. 2018). The potential use of Ti and TiO_2 for various material modifications in implantology is described in this chapter. Ti and Ti alloys, as representatives of metallic biomaterials, are recognized as the most promising materials for biomedical applications (Kaur and Singh 2019, Verma 2020).

Their leading features include high tensile strength and flexibility, corrosion resistance, and good biocompatibility. For these reasons, they have been used in implantology. Ti itself does not have antibacterial properties, so it is necessary to modify its surface to minimize the bacterial population on Ti (Tao et al. 2020). Its

hydrophobicity and low surface wettability have an adverse effect on cell adhesion in biomedical applications and that is why it is a major disadvantage (Ferreira et al. 2019).

For effective osseointegration, it is necessary to cover the surface of native Ti with a layer of hydroxyapatite, which is the main component of bone. This coating facilitates the chemical bond between implanted material and living tissue (Raphel et al. 2016). Another approach to improve the surface properties of NMs is the Atomic Layer Deposition (ALD), where the surface of the material is modified with one or more layers of another material, i.e. metal oxide (Fig. 10), which is directly correlated with the improvement of metal surface properties (Tao et al. 2020).

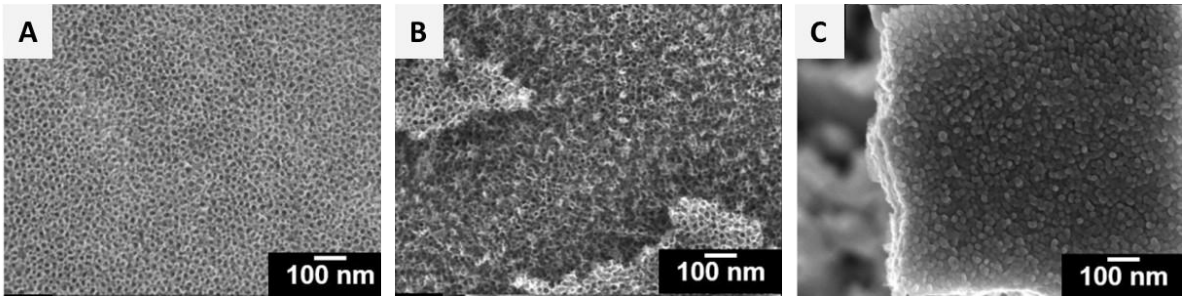

Fig. 10: Top view images of TiO_2 nanotube layers coated by Atomic Layer Deposition; (A) uncoated nanotube layer, (B) 5 cycles of TiO_2 and (C) 150 cycles of TiO_2, taken using scanning electron microscopy (Adapted from Report #5).

1.2.4.1 Principle of Atomic Layer Deposition

Generally, the Atomic Layer Deposition is a technique that can deposit various thin film materials from the gas phase. The principle of ALD was discovered in the 1960s. Gradually, the choice of materials for ALD increased and the applications expanded from bioimplantology (Fig. 11) to photovoltaics, catalysis, and semiconductor devices (Puurunen 2014).

During the ALD process, sequentially alternate pulses of gaseous chemical precursors occur resulting in chemisorption or surface reaction of the precursors (Hu, Lu and Feng 2021). These reactions on the surface of the material are called half reactions and are part of the synthesis of the material. For the precursor to be capable to fully react with the substrate surface, it is necessary to maintain suitable conditions, i.e. vacuum and temperature not exceeding 350 °C (Leskelä and Ritala 2002). The temperature range in which growth is saturated depends on the specific

ALD process and is called the ALD temperature window. The temperature of the ALD process should be optimized during the process (Astaneh et al. 2021).

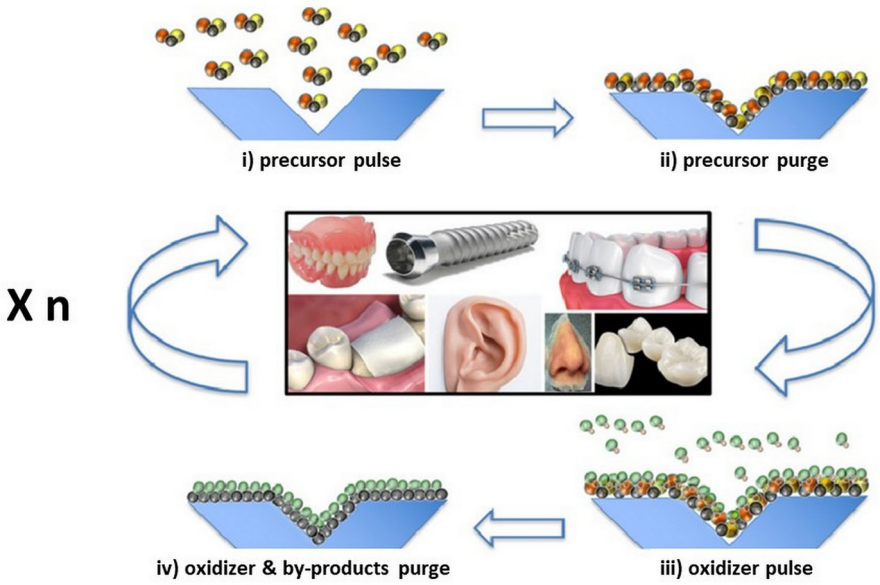

Fig. 11: : Principle of the Atomic Layer Deposition (ALD) method with potential medical applications; n = number of ALD cycles (Adapted from Astaneh et al. 2021).

When the surface of the substrate reacts with the precursor, exactly one monolayer is left. After this one-half reaction, the reaction chamber must be purged with a carrier gas, i.e., argon or nitrogen. This is followed by a pulse of the precursor against the reactants and washing forming one layer of the desired material (Johnson, Hultqvist and Bent 2014). While maintaining the correct experimental conditions, the growth is stable and the increase in thickness is constant in each application cycle (Leskela and Ritala 2002). The ALD method has shown potential advantages over alternative deposition methods, such as chemical vapor deposition (Johnson et al. 2014).

1.2.4.2 Cell growth on TiO_2-coated surfaces using Atomic Layer Deposition

The modification of the Ti implant surface using the ALD method (Tab. 3) cannot only enhance the mechanical properties of the implant but also improve the bioactivity, biocompatibility, and osteoconductivity of cells (Lu et al. 2021). The biological integration and biocompatibility of various coated surfaces have been evaluated using human cell line such as MG-63 osteoblast-like cells and HOB

primary osteoblasts (Han et al. 2016, Tian et al. 2015, Tamburaci and Tihminlioglu 2018). Murine cell lines, including L929 fibroblasts and MC3T3-E1 osteoblasts, are recommended as the standard model for biocompatibility testing (Guo et al. 2016, Radtke et al. 2019) and for assessment of pro-osteogenic properties in a variety of materials in tissue engineering studies (Wang et al. 2016, Zhang et al. 2009). The selection of the cell line directly corresponds to the cell composition of the tissue in which the metal graft is implanted.

Numerous studies show a positive effect on cell growth and proliferation by the decorating of surfaces by Atomic Layer Deposition (Tab 3). Various options for the preparation of nano titanium-based biomaterials, including severe plastic deformation, chemical etching, and ALD technique were compared (Nazarov et al. 2015). Interestingly, nature of the etching medium and the etching time have a significant qualitative impact on the nano Ti surface structure. It was suggested that increasing nanoscale roughness and greater surface hydrophilicity could contribute to improving protein adsorption, which may affect cellular activities. The relevant morphological parameter that regulates the protein adsorption process is the shape of the nanometric pores (Scopelliti et al. 2010, Yang et al. 2017).

In addition to improving the bioactivity and biocompatibility of materials, there is a need to focus on the corrosion rate of implants. Their high corrosion rate can limit their practical use, as implants can potentially corrode before the complete healing process (Chakraborty Banerjee et al. 2019). Moreover, the TiO_2 films confirmed the high potential to improve the corrosion resistance of magnesium alloys (Huang et al. 2019, Kania, Szindler and Szindler 2021).

Furthermore, the adhesive and spreading properties of human osteoblasts MG-63 in the presence of nano- and microscale structures and crystalline TiO_2 on the surface of samples with 400 cycles of ALD were improved (Nazarov et al. 2015). Etching in a basic piranha solution with a subsequent ALD of 400 TiO_2 cycles significantly stimulated implant osseointegration for MC3T3-E1 cells and increased hydrophilicity, changing the surface composition compared to nonmodified ultrafine-grained Ti samples (Nazarov et al. 2018). 2500 cycles of TiO_2 by ALD coated on Ti substrates also caused stimulation of osteoblast adhesion and proliferation compared to uncoated materials (Liu, Bhatia and Webster 2017a).

Tab. 3: Atomic Layer Deposition of titanium-based materials tested *in vitro.*

ALD film	Substrate material	Number of cycles	Thickness [nm]	Cell type	Incubation time	Outcomes	Ref.
TiO_2	Ti	5;150	0.275; 8.25	WI-38 SH-SY5Y MG-63	24 h	↑ Number of cells	(Report #5)
TiO_2	Chitin	50-400	18 (400c)	MC3T3-E1 NIH3T3	7 d	↑ Osteointegr., immunosuppres. effect	(Choy et al. 2019)
Pt / TiO_2	Collagen	200;400	27.8	Human gingival fibroblasts	7 d	Limited toxic effect on the cells	(Bishal et al. 2020)
TiO_2 / silane	AZ31 Mg	200;400	64 (400c)	MC3T3-E1	7 d	↑↑ viability, ALP	(Huang et al. 2019)
TiO_2	Ti	400	20	MG-63	5 d	↑ cell adhesion and spreading	(Nazarov et al. 2015)
TiO_2	Ultra-fine-grained Ti	400	20	MC3T3-E1	7 d	↑ proliferation, ↑ ALP	(Nazarov et al. 2018)
TiO_2	Ti	2500	100	HOB, human dermal fibroblasts	5 d	Adhesion and proliferation: ↑HOB, ↓fibroblasts	(Liu et al. 2017a)

The number of ALD cycles plays an important role in the modification of the Ti surface with TiO_2 by the ALD technique. A relatively high number of ALD cycles can provide certain disadvantages. The material can lose its original structure and become fully covered by additional coating. Furthermore, the risk of delamination can occur due to the low adhesion of the rather thick ALD coatings to the substrate, other aspects are environmental and economic disadvantages (Johnson et al. 2014).

In our Report #5, the new interest was that Ti sheets with a native oxide layer or with a crystalline thermal oxide layer and Ti nanotube layers with defined inner diameters of 12 and 15 nm were additionally coated by 5 or 150 TiO_2 cycles using ALD. We evaluated cell adhesion and growth in the response to uncoated and ALD TiO_2-coated surfaces of Ti sheets and TiO_2 nanotube (TNT) layers using fibroblast WI-38 cell line (i.e., counting of cells to quantitate their occurrence per mm^2). The

addition of 5c TiO_2 ALD was more beneficial for WI-38 cell growth than that of 150c ALD. Coating of Ti sheets and TNT layers by 5c TiO_2 ALD increased the WI-38 cell growth by >50% compared to that of the uncoated ones (Fig. 12).

We can ascribe increased cell growth on these surfaces to the additional thin 5c TiO_2 ALD coating (nominal thickness of approx. 0.3 nm) that covers the surface of Ti sheets and TNT layers by a protective TiO_2 coating with high purity and strong adhesion to the original surface. Therefore, the naturally occurring surface contaminants of Ti sheets and TNT layers are not in contact with the surface/vicinity interface with cells inducing increased WI-38 cell adhesion and growth.

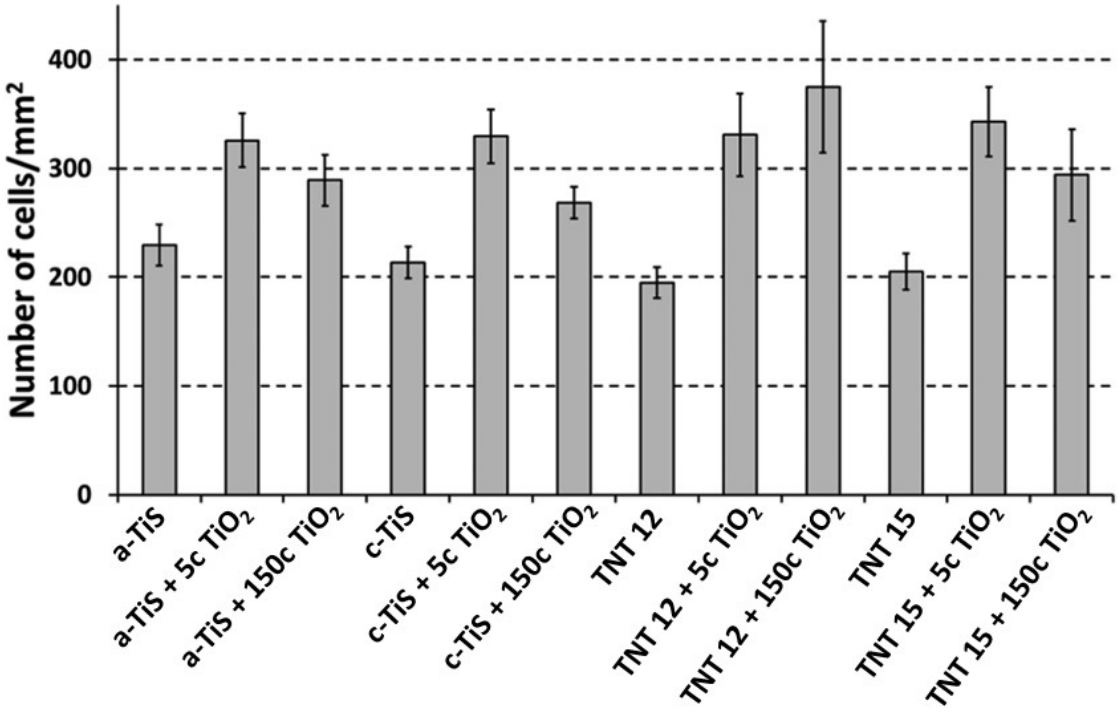

Fig. 12: Density of WI-38 cells grown for 24 h on uncoated and ALD-coated (5c and 150c TiO_2) **Ti sheets with a native oxide layer** (a-TiS), **Ti sheets with a crystalline thermal oxide layer** (c-TiS), **and TiO_2 nanotube layers** with inner diameters of 12 and 15 nm (TNT 12 and TNT 15). Data are presented as mean ± SEM (Report #5).

In our Report #5, the WI-38 cell growth was increased on Ti sheets and TNT layers after coating by 150c TiO_2 ALD compared to their uncoated counterparts, but it was not so high compared to the WI-38 cell growth on Ti sheets and TNT layers coated by 5c TiO_2 ALD. Moreover, the cell growth of cell lines with a different origin and morphology on uncoated and ALD 5c TiO_2-coated TNT layers was evaluated.

The cell growth of SH-SY5Y and MG-63 cells after 24 h of incubation increased by >30% on 5c TiO_2-coated TNT layers compared to that of the uncoated ones.

In general, this novel ALD-based approach offers distinct advantages. Ti sheets and TNT layers coated by additional 5c TiO_2 ALD retain their original structure. This is especially demanded for TNT layers with their unique morphology. A thin TiO_2 ALD coating protects the TNT layers from crystallization and shape change due to water annealing and prevents contaminants from being in a direct contact with cells. The risk of delamination of the additional TiO_2 ALD coating is avoided due to the nature of the ALD process creating strong chemical bonds between substrates and coatings. Our results pave a way for the modification of surfaces using various ALD-coatings to increase their biocompatibility and promote cell growth.

2. Objectives of the thesis

The development and production of new nanomaterials have been increasing in recent years including the use of nanomaterials for various purposes based on their unique properties. However, some issues can occur within the testing of biological effects of nanomaterials *in vitro*. Therefore, it is crucial and indispensable to characterize nanomaterials, agglomeration, evaluate range of doses, and behavior of nanomaterials in model systems for biological testing *in vitro*. The aims of the PhD thesis were addressed in five reports presented in the chapter 3.

The objectives of the thesis were:

1. To optimize and validate TiO_2 P25 nanoparticle preparation for biological testing in cultured pulmonary cells and to evaluate their biological effects **(Report #1).**

2. To characterize the biological effects of nanomaterials of different shape and chemical composition, i.e., inorganic nanoparticles vs. fibers **(Report #2)** and other TiO_2-derived nanomaterials **(Reports #3 and #4)** in cultured pulmonary cells.

3. To estimate the effect of TiO_2 Atomic Layer Deposition coating of materials on their biocompatibility in cells *in vitro* **(Report #5).**

In this thesis, also three other studies with my co-authorship were included because they estimated the cell cultivation and toxicity. These reports have been focused on a study of the effect of passaging on the susceptibility of cells to toxic compounds **(Report #6)**, the toxicity of new organic compounds **(Report #7)**, and influence of bioactive ions on osteoclast formation *in vitro* **(Report #8)**.

3. Published studies

3.1 Reports related to testing of nanomaterials

3.2 Other published reports