Journal of Breath Research

PAPER

Raman microspectroscopy of exhaled breath condensate and urine in workers exposed to fine and nano TiO_2 particles: a cross-sectional study

RECEIVED
7 February 2015

REVISED
20 May 2015

ACCEPTED FOR PUBLICATION
8 June 2015

PUBLISHED
15 July 2015

Daniela Pelclova[1], Hana Barosova[2], Jana Kukutschova[2], Vladimir Zdimal[3], Tomas Navratil[4,5], Zdenka Fenclova[1], Stepanka Vlckova[1], Jaroslav Schwarz[3], Nadezda Zikova[3], Petr Kacer[6], Martin Komarc[7], Jaroslav Belacek[7] and Sergey Zakharov[1]

[1] Charles University in Prague and General University Hospital in Prague, First Faculty of Medicine, Department of Occupational Medicine, Na Bojisti 1, 128 00 Prague 2, Czech Republic
[2] Nanotechnology Centre, VSB—Technical University of Ostrava, 17. Listopadu 15, 708 33 Ostrava, Czech Republic
[3] Institute of Chemical Process Fundamentals of the AS CR, v.v.i., Rozvojova 1/135, 165 02 Prague 6, Czech Republic
[4] J. Heyrovsky Institute of Physical Chemistry of the AS CR, v.v.i., Dolejskova 3, 182 23 Prague 8, Czech Republic
[5] Charles University in Prague and General University Hospital in Prague, First Faculty of Medicine, Institute of Medical Biochemistry and Laboratory Diagnostics, Katerinska 32, 121 08 Prague 2, Czech Republic
[6] Institute of Chemical Technology, Technicka 5, 166 28 Prague 6, Czech Republic
[7] Charles University in Prague and General University Hospital in Prague, First Faculty of Medicine, Institute of Biophysics and Informatics, Salmovska 1, 120 00 Prague 2, Czech Republic

E-mail: daniela.pelclova@lf1.cuni.cz

Keywords: nanoparticles, TiO_2, titanium, exhaled breath condensate, urine, occupational exposure, Raman microspectroscopy

Supplementary material for this article is available online

Abstract

The health effects of engineered nanoparticles in humans are not well-understood; however experimental data support the theory of oxidative stress promoting fibrogenesis and carcinogenicity. The aim of this study was to detect TiO_2 particles in exhaled breath condensate (EBC) and urine samples to ascertain their presence and potential persistence and excretion in urine.

EBC and urine samples were collected from 20 workers exposed to TiO_2 aerosol; among them, 16 had a higher risk level of exposure (production workers) and four had medium risk level (research workers); in addition to 20 controls. Titanium levels in EBC and urine were analysed using the inductively coupled plasma mass spectrometry (ICP-MS) method. A Raman microspectroscopic analysis was performed in EBC and urine to identify the phase composition of TiO_2 particles observed. Aerosol exposure in the workplaces was measured using SMPS and APS spectrometers and P-TRAK and DustTRAK DRX monitors.

The median concentration of TiO_2 aerosol was 1.98×10^4 particles cm^{-3}, the interquartile range (IQR) was $1.50 \times 10^4 - 3.01 \times 10^4$ particles cm^{-3} and the median mass concentration was 0.65 mg m^{-3} (IQR 0.46–.0.83 mg m^{-3}); 70–82% of the particles were smaller than 100 nm in diameter. In any part of the plant, the median TiO_2 air concentration did not exceed the national airborne exposure limit of 10 mg m^{-3} for inert dust. Particles of rutile and/or anatase were found in the EBC of exposed workers in 8/20 (40%) of the pre-shift and 14/20 (70%) of the post-shift samples. In the urine of workers, TiO_2 particles were detected in 2/20 post-shift urine samples only. The mean concentration of titanium in the EBC in production workers was 24.1 ± 1.8 μg/l. In the research workers the values were below the limit of quantitation; LOQ = 4.0 ± 0.2 μg/l), as well as in the controls. In the urine samples of all of the subjects, titanium was under the limit of detection (LOD = 1.2 μg/l). Raman microanalysis of EBC in the workers confirmed the presence of TiO_2 anatase and/or rutile crystal phases in the pre-shift samples and their persistence from previous shifts in the workers.

Abbreviations

APS	aerodynamic particle sizer
DNA	deoxyribonucleic acid
EBC	exhaled breath condensate
FeNO	fractional exhaled nitric oxide
FEV1	forced expiratory volume at 1s
FVC	forced vital capacity
IARC	International Agency for Research on Cancer
ICP-MS	inductively coupled plasma mass spectrometry
IQR	interquartile range
LOD	limit of detection
LOQ	limit of quantification
NIOSH	National Institute for Occupational Safety and Health
PSD	particle number size distributions
RL	risk level
RNA	ribonucleic acid
ROS	reactive oxygen species
SMPS	scanning mobility particle sizer
SPE	solid-phase extraction
UV	ultraviolet

1. Introduction

Potential adverse health effects of engineered TiO_2 nanoparticles in humans are not yet well-understood. Since their surface properties, energy level, electronic structure, and reactivity are substantially different from the properties of the coarse particles of the same chemical composition, they may possess dramatically different properties and their toxicity is supposed to be higher [1–3].

TiO_2 (CAS No. 13436-67-7), also known as titanium oxide, titania or Ti white, is a non-combustible and odourless poorly soluble powder that has been widely used as a white pigment because of its brightness and opacifying strength. It is permitted in the food industry as an additive (E171), and is also permitted in the pharmaceutical industry. In sunscreens, it is used as an ultraviolet ray scavenger, in addition to reflecting rays away from the skin [4, 5].

Anatase, rutile and brookite are three crystal polymorphs of TiO_2. In particular, the rutile and anatase phases of nanosized TiO_2, available in variable sizes, shapes and coatings, are increasingly utilised in many applications. Based on numerous experimental studies, anatase is known to be most chemically reactive and toxic [2, 6]. It generates six-fold more reactive oxygen species (ROS) than does rutile after UV irradiation. However, in the nanosized (10 and 20 nm) form, anatase induced oxidative DNA damage, lipid peroxidation and micronuclei formation in a human bronchial epithelial cell line, even in the absence of photoactivation [7, 8]. Rutile, on the contrary, is believed to be chemically inert; however under certain conditions (without photoactivation), a mixture of rutile-anatase leads to a higher level of oxidative DNA damage than does anatase alone [7, 8].

Experimental studies highlight the proof of biological effects of TiO_2 nanoparticles on the respiratory system with the generation of oxidative stress, pro-inflammatory effects and the possible development of fibrosis, pulmonary emphysema and/or cancer [2, 6]. The inhaled agglomerates of TiO_2 are not stable in body fluids; they may dissociate to nanoparticles and be transported from the lungs to other organs [2]. It is known that the elimination of TiO_2 coarse particles from the lungs is very slow; however there are no data about fine (between 100 and 2,500 nm in size) or nano (less than 100 nm in size) TiO_2 particle excretion in the urine of humans [9, 10].

Exhaled breath condensate (EBC) is the liquid obtained after cooling the exhaled air of a subject, supposedly reflecting the composition of the bronchoalveolar lining fluid [11, 12]. The sampling method is non-invasive, which makes it suitable for the detection of biomarkers due to occupational inhalational exposure [13], including carcinogenic and fibrogenic dust, i.e. silica and asbestos [14, 15], where oxidative stress plays an important role in the pathogenesis of lung fibroses and cancer [16, 17].

Based on the experimental data that Møller *et al* reviewed, humans exposed to engineered nanomaterials may have higher levels of oxidative damage [16]. This hypothesis was supported by our pilot study of EBC and urine markers of oxidative/nitrosative stress in workers exposed to TiO_2 nanoparticles. In both pre-shift and post-shift EBC samples, the examined biomarkers were significantly higher ($p < 0.001$) compared to the controls. They included oxidation markers for lipids, proteins and nucleic acids. Several markers of inflammation, such as leukotrienes B_4, C_4, E_4 (all $p < 0.001$) and fractional exhaled nitric oxide (FeNO) ($p < 0.05$) were increased in the EBC [18].

TiO_2 fine particles can be detected and the phase composition may be specified in tissue samples, bronchoalveolar lavage fluid or in other biological samples using Raman microspectrometry. The method allows for visualising the particles that have a diameter of 100 nm or more [19, 20]. Raman microspectroscopy was also successfully used to reveal the cause of mica-caused diffuse pulmonary fibrosis with impairment of respiratory function, thanks to the discovery of mica particles in the EBC of a worker occupationally exposed to mica dust in a grinding factory for seven years [19, 20].

According to the International Agency for Research on Cancer (IARC), TiO_2 is classified as possibly carcinogenic to humans, group 2B [21]. The multicountry European study (six countries) of TiO_2 production workers found a slightly increased risk for lung cancer and a suggestive dose-response risk for kidney cancer [22]. There is no doubt that the potentially damaging health effects of persistent TiO_2 nanoparticles in the respiratory system of humans warrant careful work-

place monitoring, as well as efforts to lower exposure to prevent possible late effects [23]. Nanoparticles were selected to be a priority for occupational safety and health research in Europe for the years 2013–2020 [24]; however, very little human data have been presented thus far. As a precautionary step, NIOSH has recommended 0.3 mg m^{-3} limit for ultrafine (including engineered nanoscale) TiO_2 [23].

The aim of this study was to detect TiO_2 particles in EBC and urine samples and to specify their anatase or rutile crystal phases using Raman microspectroscopy. Additionally, the potential association between the age, length and severity of exposure, smoking status and spirometry parameters with the presence of TiO_2 crystals and characteristics of EBC sample, such as its pH, conductivity and titanium concentration, was studied.

2. Methods

2.1. Aerosol measurements

TiO_2 pigment in the plant is produced from titanium mineral ilmenite by the sulphate process. After the reaction with sulphuric acid, titanium hydroxide is then precipitated by hydrolysis, filtered and calcined. During calcination, the material is heated to 800–1000 °C and the anatase/rutile crystals are formed. In the finishing operations, the crude form of the pigment is milled (micronisation) to produce a controlled distribution of particle size and the surface is treated to improve its functional behaviour.

The dynamics of aerosol particle number size distributions (PSD) in the workplace were monitored by a scanning mobility particle sizer (SMPS), model 3936 L, TSI, USA, as well as an aerodynamic particle sizer (APS), model 3321, TSI, USA. These aerosol spectrometers sampled synchronously with a 5 min time resolution, covering the overall particle size range from 15 nm to 10 μm in diameter, with a size resolution of 32 channels/decade. Simultaneously, the spatial distributions of the total particle number and mass concentrations, respectively, were determined using a portable particle number concentration monitor (P-TRAK) and a portable monitor of particle mass concentrations (DustTRAK DRX), respectively. During the shifts, the measurements of highly time- and size-resolved aerosol concentrations were carried out. At first, pilot measurements have been carried out for mapping and localisation of the main sources of aerosol particles using P-TRAK and DustTRAK DRX to construct the concentration maps and to find the key locations in the workplace. The air samples were taken in more than 30 sites of the plant. The spectrometers Scanning Mobility Particle Sizer™ Spectrometer 3936 (SMPS) and Aerodynamic Particle Sizer® Spectrometer 3321 (APS) have then been used for continuous monitoring of particle size distribution during the whole 8 h shift with 5 min sampling frequency. The samples were collected based on placing the samplers 1–4 m away from the manufacturing equipment. For the 8 h shift sampling, four locations have been selected: micronisation, calcination furnace, transport corridors, and the control room. These four locations were seen as the most representative for the group of the examined workers. For each shift the spectrometers were placed at another location. During the measurements a random checks were performed to compare the total number concentrations determined by SMPS with the P-TRAK values (the differences between averaged values never exceeded 20%). Similarly the PM_{10} mass concentrations determined by the DustTRAK DRX were compared with the PM_{10} integrated from the APS data assuming particles density corresponding to TiO_2 (4 g ccm^{-1}). While the workers spent most of the shift time in the control room, separated by a closed door, they were working in the close vicinity of one of the three of particle emitting production units in the remaining time of the shift. All workers in the study received and used respiratory protective devices during the working operations.

2.2. Subjects

Twenty workers (males, mean age 33.7 ± 4.9 years, 11 smokers, 9 non-smokers) exposed to TiO_2 were studied. The mean length of TiO_2 exposure was 9.1 ± 3.7 years. Workers had to meet two criteria: (1) male gender; (2) working with TiO_2 for at least 6 months. The nanoparticle exposure was classified according to the control banding nanotool risk level (RL) matrix. The matrix is constituted from the severity factor of the material itself (70% of the score is formed by the nanomaterial characteristics, such as its solubility, particle shape, size, and experimental toxicity; in addition to 30% of the score due to the characteristics of the parent material, its occupational exposure limits and human toxicity). Secondly, the probability score of exposure is evaluated based on the amount of material used, air concentration, and frequency and duration of the operations [25]. The evaluation is given in supplemental table S1 (stacks.iop.org/JBR/9/036008/mmedia). There were four workers (Nos. 1–4) employed in the research part of the plant, where the risk level, RL was classified as medium (RL3). Furthermore, 16 workers (Nos. 5–20) were exposed to TiO_2 aerosol in the production part of the plant; their risk level of nanoparticle exposure was higher (RL4). Among them, six subjects were employed in the calcination process, four in micronisation, five in coating, and one in filtration. Measurements were performed on all workers both before and after the eight-hour shift in the first half of the week (from Tuesday to Wednesday). Twenty controls (males mean age 34.8 ± 4.6 years, nine smokers, 10 non-smokers) who were not employed in this factory and not exposed occupationally to dust or other health risks (safety inspectors and office workers) were also examined. Twelve men lived in another city and eight in the same city as the workers. Exclusion criteria for all subjects included history of tuberculosis, lung cancer, pulmonary surgery, myocarditis, congenital heart disease, and recent fever and/or inflammation.

Both groups of subjects were examined according to the following scheme: physical examination and a standardised questionnaire with personal and occupational history, treatments, and lifestyle habits (diet, smoking habits and alcohol intake).

Pre-shift and post-shift forced vital capacity (FVC%) and forced expiratory volume at 1s (FEV1%) were measured using a SpiroPro Jaeger, Germany. The pre-shift and post-shift EBC samples were collected using Ecoscreen Turbo DECCS, Jaeger, Germany equipped with a filter. All subjects breathed tidally through a mouthpiece connected to the condenser (−20 °C) wearing a nose-clip. A constant volume of exhaled air of 120 litres was maintained. The metal tube of Ecoscreen Turbo was cleaned with ethanol after every use. Besides pH, the conductivity was measured to supress the dilution factor of EBC [26]. In the workers, pre-shift and post-shift spot urine samples were collected. The controls were examined and they gave their EBC and urine samples only once.

All samples were immediately frozen and stored at −80 °C until analysis.

2.3. Titanium analysis

TiO_2 crystallographic measurements were performed using a four-circle CCD diffractometer Geminy of Oxford Diffraction, Ltd., with graphite monochromated Mo K_{alpha} radiation ($k = 0.71073$ A°). A quantitative analysis of titanium in EBC and urine was done using the ICP-MS technique. Agilent 7900 ICP-MS Ultra HMI (UHMI), equipped with MassHunter software and the autosampler ASX-520, was used. Before measurement the liquid samples were evaporated to dryness and mineralized with the mixture of HF and HNO_3 (1:3, v/v) in UniClever microwave decomposition unit (Plazmatronika-Service, Wroclaw, Poland). The method was validated and used for quantitative measurements. The limit of detection (LOD) was 1.2 μg/l and the limit of quantification (LOQ) was 4.0 ± 0.2 μg/l. The standard error was 3.0%.

2.4. Raman microspectroscopy

EBC and urine samples were treated prior to the phase analysis in laminar FlowboxSteril-BIOBAN 48 Compact. Liquid samples were dropped on a glass slide and dried at room temperature. The samples were dropped in multi-layers to get better background resolution and more particles deposited. The total volumes of the samples dropped were about 50 μl and 100 μl for EBC and urine, respectively. The samples on the glass slides were systematically analysed by visualising the entire surface of by light microscope as a part of the Smart Raman Microscopy System XploRA™ (HoribaYvonJobin, France).

All samples were analysed using the Smart Raman Microscopy System XploRA™ (HoribaYvonJobin, France), allowing for the detection and phase analysis of solid particles in analysed samples by combination of the Raman spectroscopic technique with a confocal microscope (Olympus BX51). Raman spectra were acquired with a 532 nm excitation laser source and 1200 gr. mm^{-1} grating. This method used for qualitative evaluation allowed for the analysis of submicron particles with a diameter of 100 nm or more. TiO_2 particles were evaluated as present if at least one TiO_2 containing particle was detected in the slide.

2.5. Ethics

The Ethical Committee of the 1st Medical Faculty, Charles University in Prague approved the study. All participants were informed about the aim of the study at least five days earlier. They signed the informed consent before the beginning of the study.

2.6. Statistical evaluation

Statistical evaluations were carried out using following basic statistical tests: test of normality of distribution, arithmetic mean, confidence intervals, skew, median, mode, as well as an *F*-test for equal means. Significances of normally distributed data were evaluated using Student's *t*-test for equal/unequal variances. In the non-normally distributed data, a Mann–Whitney test was used. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Aerosol measurements

Measurements in the production plant with RL4 of exposure showed that the total aerosol concentrations varied greatly in both space and time. The median number concentration (SMPS + APS) at the three monitoring positions located close to the production units was 1.98×10^4 particles cm^{-3}, with an interquartile range (IQR) of 1.50×10^4 to 3.01×10^4 particles cm^{-3}, and the median mass concentration (SMPS + APS) was 0.65 mg m^{-3} (IQR 0.46 to 0.83 mg m^{-3}). In the workshops, 70–82% of those particles were smaller than 100 nm in diameter, i.e. having the ability to penetrate deeply into the alveolar region of the respiratory tract.

In the research part of the plant with RL3 of exposure, the median number concentrations, (SMPS + APS) 1.55×10^4 particles cm^{-3} (IQR 1.35×10^4 to 1.60×10^4 particles cm^{-3}), and the median mass concentration, (SMPS + APS) 0.164 mg m^{-3} (IQR 0.148 to 0.216 mg m^{-3}), were much lower. Here, 40–70% of those particles were smaller than 100 nm in diameter. The space was ventilated from the outdoors due to an open main door, so the measured size distributions practically represent the ambient aerosol concentrations, at least in the ultrafine size range.

According to their time sheets, the workers from the production part spent about one third of their shifts in close vicinity of the particle emitting production units; the remaining two thirds of the time, they were staying in the control room and checking the production lines remotely. In the control room, the median total

number concentration was one order of magnitude lower than it was in the positions located close to the production units. The median number concentration was 0.43×10^4 particles cm^{-3} (IQR from 0.29×10^4 to 0.61×10^4 particles cm^{-3}), and the median mass concentration was (SMPS + APS) 0.13 mg m^{-3} (IQR 0.096 to 0.221 mg m^{-3}).

The highest number concentrations measured by SMPS (14–710 nm) were found in the vicinity of the calcination furnace (peak of particles 30–40 nm in diameter) which was the source of these particles. On the other hand, the size distribution of particles at the micronisation unit of the plant was flat and broad with a maximum concentration of the particles in the range of 30–40 nm as well. It showed that aerosol in the production plant formed an external mixture on its way to this area from other parts of the hall with the peak at calcination furnace. At the transport corridors, there seemed to be another source with a peak size between 20 and 30 nm. In the control room, the median particle size distribution was very different and indicated a completely other aerosol originating mainly outdoors, peaking in the accumulation mode close to 100 nm.

Measurement using APS (0.5–10 μm) confirmed the lowest number concentrations in the control room by almost one order of magnitude. The highest concentrations were found again at the calcination furnace, they were in the range 700–800 nm. Particles of the same size at the micronisation unit had twofold lower concentration, and at transport corridors about fourfold lower concentration, which suggests the transport across the hall. On the other hand, the micronisation process probably emitted particles with diameter under 2 μm and around 6 μm as their concentrations were higher than in other measured locations.

Concerning the source of exposure in the control room, most of aerosol particles leaked indoors from the ambient air of the production plant, and the majority of these particles was found in the accumulation mode centred around 100 nm in particle diameter. The median size of these particles was 93 nm, IQR from 54 to 153 nm. It is worth noting that in any part of the plant, the medium TiO_2 air concentration in the workplace did not exceed the national allowed concentrations for inert dust (10 mg m^{-3}), however it might have potentially exceeded NIOSH recommended airborne exposure limit of 0.3 mg m^{-3} for ultrafine TiO_2 particles in the production part of the factory [23].

3.2. Raman microspectroscopy

In the EBC of the exposed workers, the rutile and/or anatase particles were found in 22/40 (55%) samples; in the pre-shift measurements, they were found in 8/20 (40%) workers and in the post-shift measurements, they were found in 14/20 (70%) workers, as shown in table 1.

The TiO_2 crystals were present in 50% of the EBC samples from Nos. 1–4 RL3 workers as well (in 25% pre-shift and 75% post-shift samples). Anatase was identified more frequently (18 samples) than was rutile (15 samples), and 12 samples contained both crystal structures. Examples of anatase and rutile TiO_2 crystals detected by Raman microspectrometry are presented in figures 1–3. Additionally, in the EBC of the workers, calcite was found in 75%, carbon in 20% and SiO_2 in 10% of the samples; hematite and nitrates were found in 5%, as shown in table 1. Anatase/rutile crystals were not found in the EBC samples of the control subjects. Additionally, other inorganic particles were less frequent in the EBC of the controls; calcite was found in 20%, carbon in 10% and SiO_2 in 5% of the samples.

In the urine samples of the workers, anatase particles with a diameter greater than 100 nm were found in two (10%) workers post-shift (Nos. 1 and 9), as shown in table 1. Their ages were 32 and 31 years, and they were employed at the plant for four and seven years, respectively. Both were smokers. Calcite and barite were found in 10% of the post-shift urine samples. The analysis of the controls' urine using Raman microspectrometry was completely negative, and no inorganic particles were found.

3.3. Titanium analysis in EBC and urine

The results of titanium measurements in the EBC of the 16 production (RL4) workers and controls are shown in table 2. The mean titanium concentration in EBC samples of four research (RL3) workers (No 1–4) was at the LOQ $4.0 \pm 0.0\,\mu g\,l^{-1}$. In the controls, the concentration was ⩽LOD, only one subject had the concentration at the LOQ. There was no statistical difference in the mean concentration of titanium between the workers with and without detected TiO_2 crystals in the EBC samples, both in the pre-shift (22.8 ± 6.9 versus $18.3 \pm 5.7\,\mu g\,l^{-1}$, $p = 0.295$) and in the post-shift EBC samples (19.8 ± 5.2 versus $20.8 \pm 9.9\,\mu g\,l^{-1}$, $p = 0.826$). In the urine of both the exposed workers and the controls, the titanium concentration was under the LOD of the method.

3.4. pH and conductivity of EBC samples

The acidity of the EBC was significantly higher in the production workers, both in the pre-shift and in the post-shift samples compared to the samples of the controls, as can be seen in table 2. There was no difference in the mean pH of the samples of the workers with and without TiO_2 crystals in the pre-shift samples (4.68 ± 0.53 versus 4.93 ± 0.27, $p = 0.321$). Similarly, no difference was found for pH of the post-shift EBC samples with and without crystals (4.89 ± 0.34 versus 5.13 ± 0.48; $p = 0.407$).

The conductivity of the EBC samples of RL4 workers and controls is presented in table 2; it did not differ significantly between the workers and the controls.

3.5. Spirometry

The spirometry parameters FVC% and FEV_1% did not differ between RL4 exposed workers and controls, as seen in table 2.

Table 1. Particles found in exhaled breath condensate and urine in the TiO_2 exposed workers (research workers Nos. 1–4 with risk level 3, production workers with risk level 4).

	Exhaled breath condensate		Urine		
Worker no.	Pre-shift	Post-shift	Pre-shift	Post-shift	Work operation
1	Anatase, calcite, carbon, SiO_2	Anatase, calcite	—	Anatase, barite	research
2	Calcite	Anatase, rutile	—	—	Research
3	—	—	—	—	Research
4	—	Rutile	—	—	Research
5	Calcite	Anatase, rutile, carbon	—	—	Micronisation
6	Anatase, rutile, calcite, carbon, SiO_2	Anatase, rutile, calcite	—	—	Calcination
7	Rutile, calcite	Anatase, rutile	—	Calcite	Calcination
8	—	Anatase, rutile, calcite	—	Barite	Coating
9	Anatase, rutile, calcite	Anatase, rutile, hematite	—	Anatase, calcite	Calcination
10	Calcite	Calcite	—	—	Calcination
11	Calcite	NO_3-nitrates	—	—	Coating
12	Anatase, rutile, carbon (Figure 1)	—	—	—	Micronisation
13	Anatase, calcite	Anatase	—	—	Coating
14	Anatase, rutile	Anatase, rutile (Figures 2 and 3)	—	—	Calcination
15	—	Calcite	—	—	Coating
16	—	Anatase	—	—	Calcination
17	—	Rutile	—	—	Micronisation
18	Calcite	Rutile	—	—	Micronisation
19	Anatase	Anatase	—	—	Coating
20	Carbon	—	—	—	Filtration

Table 2. Titanium and conductivity in exhaled breath condensate samples and spirometry parameters of production workers and controls. FVC% = forced vital capacity, FEV1% = forced expiratory volume at 1s. Mean and 95% confidence interval are presented.

	Workers Pre-shift ($N = 16$)	Workers Post-shift ($N = 16$)	Controls ($N = 20$)	P (Pre-shift versus Controls)	P (Post-shift versus Controls)	P (Pre-shift versus Post-shift)
Ti in EBC [$\mu g l^{-1}$]	24.1 ± 1.8	24.1 ± 1.9	Not quantified	—	—	0.961
Conductivity of EBC [$\mu S\,cm^{-1}$]	127 ± 23	122 ± 17	115 ± 22	0.435	0.602	0.728
pH of EBC	4.77 ± 0.30	4.81 ± 0.27	5.74 ± 0.67	0.012[a]	0.015[a]	0.842
FVC [% predicted]	104.8 ± 6.2	103.0 ± 5.8	104.3 ± 7.2	0.915	0.783	0.661
FEV1 [% predicted]	83.4 ± 2.6	83.1 ± 2.4	82.6 ± 3.1	0.687	0.773	0.877
FEV1/FVC	0.806 ± 0.059	0.816 ± 0.053	0.815 ± 0.084	0.865	0.979	0.799

[a] $p < 0.05$.

4. Discussion

The method of Raman microspectroscopy of the EBC samples allowed for the detection of anatase and rutile crystal forms of TiO_2 in exposed workers from different parts of the production to TiO_2 white pigment. The transport of the particles originating from the calcination process across the production hall could be tracked by aerosol measurements and distinguished by particles size from the aerosols in the transport corridors. The crystals were detected not only in the workers at a higher risk category (RL4), but also in workers Nos. 1–4 from the research part of the factory with a lower risk level category (RL3). Anatase and rutile have not been found in the EBC samples of the controls, as had been expected.

Raman spectrum has been analysed and compared with the spectrum library and literature data [27–30] and show similar spectrum also with the collected dust samples. It is known that the presence of other components in the sample may slightly affect the spectrum and Raman bands of biological samples. Therefore, a completely identical spectrum cannot be expected. Similarly, the spectrum in the samples obtained from the environment can be slightly shifted in comparison to library spectrum of chemically clean material.

pH of the EBC was lower in the exposed workers, this finding can be associated with the inflammation in the airways, as had been described in the patients with asthma and chronic bronchitis [31].

Detection of anatase and/or rutile crystals in the EBC by Raman microspectroscopy was not associ-

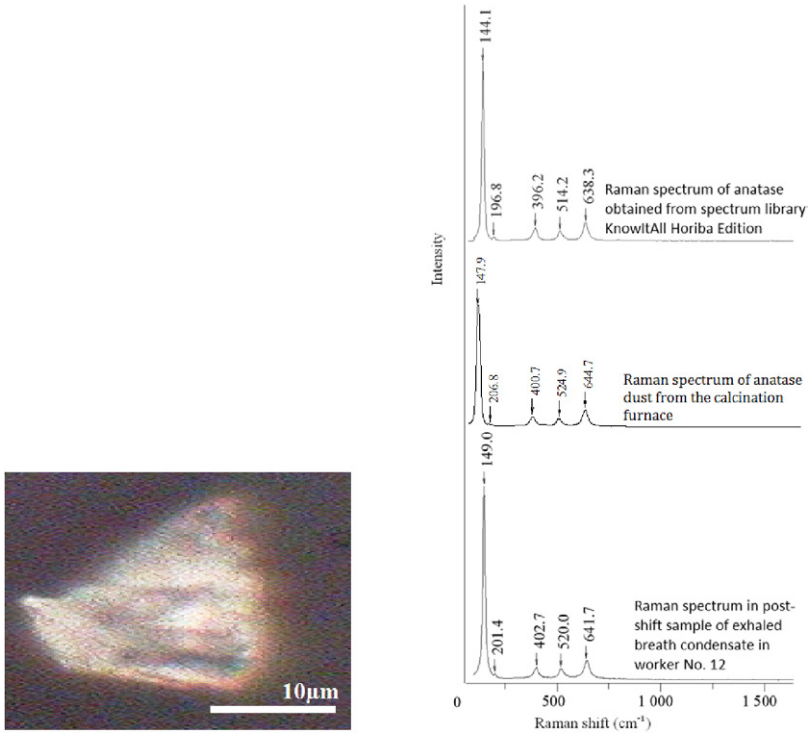

Figure 1. Anatase crystal structure (left) and Raman spectra (right). Post-shift sample of exhaled breath condensate in a worker from TiO_2 production (lowest spectrum) shows peaks corresponding to Raman spectrum of anatase dust from the vicinity of calcination furnace (middle spectrum) and Raman spectrum of anatase obtained from the spectrum library KnowItAll Horiba Edition (upper spectrum).

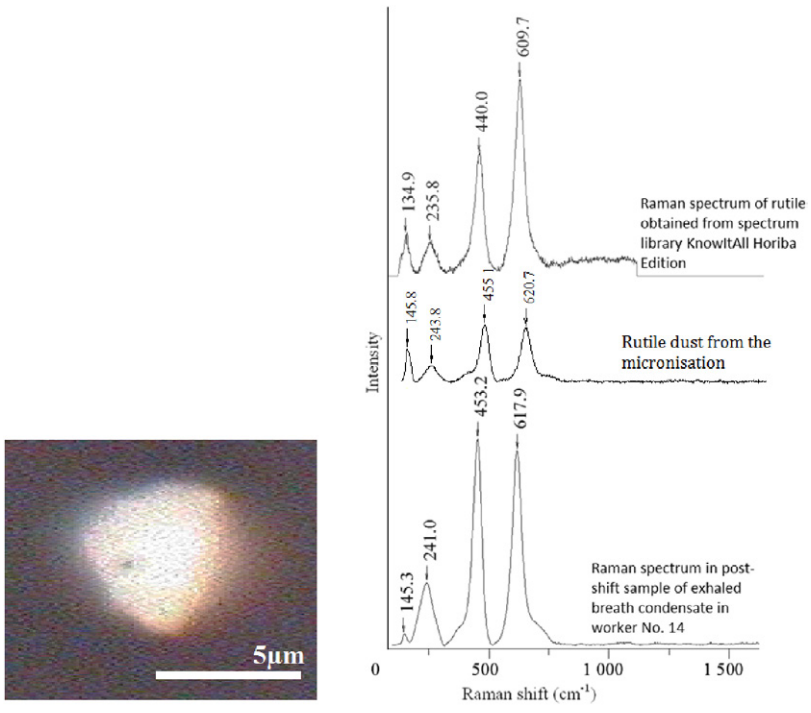

Figure 2. Rutile crystal structure (left) and Raman spectra (right). Post-shift sample of exhaled breath condensate in a worker from TiO_2 production (lowest spectrum) shows peaks corresponding to Raman spectrum of rutile dust obtained after the micronisation process (middle spectrum) and Raman spectrum of rutile from the spectrum library KnowItAll Horiba Edition (upper spectrum).

ated with elevated concentrations of titanium, different acidity or conductivity of the EBC sample of exposed workers. This discrepancy may be explained by the fact that TiO_2 crystals finding could be related to a more recent and probably short-term exposure before the elimination of the particles by the mucociliary system. On the other hand, elevated titanium concentration in the EBC of the workers may reflect a

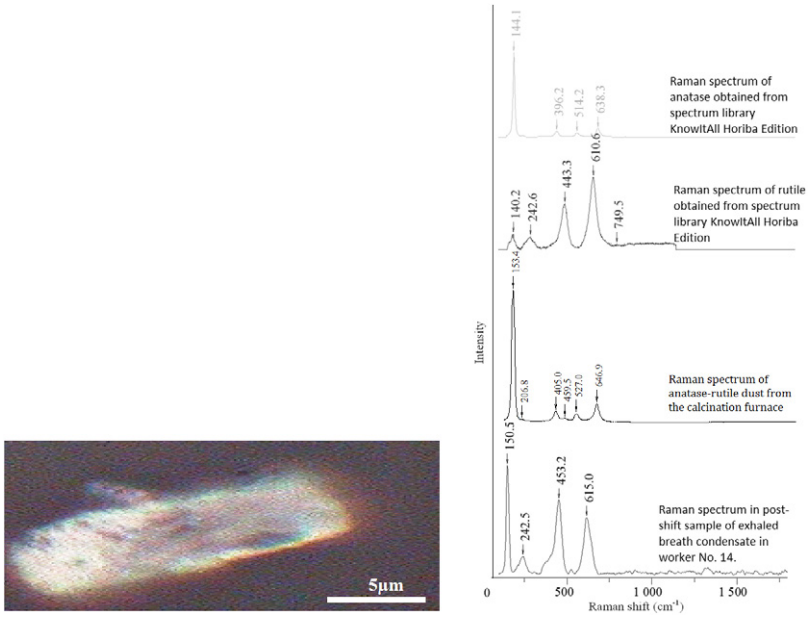

Figure 3. Anatase and rutile crystal structures (left) and Raman spectra (right). Post-shift sample of exhaled breath condensate in a worker from TiO_2 production (lowest spectrum) with peaks corresponding to Raman spectrum of anatase and rutile shows that the particle is composed from mixture of anatase and rutile crystal phase. Raman spectrum of anatase and rutile obtained from the calcination furnace (higher spectrum) show peaks corresponding to the lowest spectrum. The two upper Raman spectra show the spectrum of anatase and rutile from the spectrum library KnowItAll Horiba Edition.

long-term persistence in the respiratory system of the residual nanoparticles at the smaller size than observable level with Raman microspectroscopy. The increase of the anatase/rutile particles in the post-shift Raman analysis and stable titanium concentration in EBC may support this hypothesis. In addition, Raman analysis did not give quantitative information. Therefore, a correlation with titanium level in the EBC cannot be expected, as one particle finding was counted as a positive finding.

The persistence of TiO_2 particles in the respiratory system supports the hypothesis of the generation of oxidative stress, proven by significantly elevated oxidation markers for lipids, proteins and nucleic acids in EBC samples collected from these exposed workers, compared to the control group, as shown in our pilot study [18]. Importantly, it was found that the anatase/rutile particles were already present in the respiratory system before the shift, i.e. they persisted from previous shift or several shifts. This was in agreement with the findings of both significantly elevated titanium concentrations in pre-shift EBC samples and the pre-shift markers of oxidative stress in that pilot study. Such data would be rather alarming, as oxidative stress induced by TiO_2 nanoparticle exposure in experimental studies has been associated with their toxic and carcinogenic effects [2, 32].

The question concerning the length of persistence of anatase/rutile crystals in the EBC samples, and the question of the length of occurrence and reversibility of the oxidative stress caused by TiO_2 nanoparticles cannot yet be answered. Some studies report transient and more moderate pulmonary inflammatory/cytotoxic indices that persisted only one month post-exposure [4].

Similarly, Kwon *et al* [33] observed histopathological changes in the nasal mucosa of rats after exposure to nano TiO_2 (anatase and rutile phase) for two weeks (six hours/day, five days/week) at a mean mass concentration approximately 11 mg m^{-3}. The exposure to 20 nm particles resulted in toxicity, even if the total particle number was relatively low. Interestingly, these lesions were observed at post-exposure days one and seven, and resolved at day 15. In this study, the degree of toxicity induced by TiO_2 nanoparticles correlated with the delivered quantities. These data might suggest the potential of lowering the effects of nanoparticles in workers by decreasing not only the whole dust burden, but especially the workplace exposure to nanoparticles, to a lower level.

Another noticeable result of our study was the absence of TiO_2 crystals in the pre-shift urine in majority of the workers. Detection of anatase in post-shift urine samples of two workers may be explained by contamination while voiding urine. On the other hand, the possibility of the transport from the blood to the kidneys cannot be excluded, as they may enter the GI tract; either from ingestion due to the contamination of the hands or from particles cleared out from the lungs by mucociliary transport [34]. TiO_2 fine particles have been shown to be absorbed from the GI tract of the rat and systematically translocate to other tissues; however data concerning humans are not available [2].

Also, other inorganic particles ($CaCO_3$, SiO_2) were detected in the urine of the workers only with the post-

shift measurements, which might have originated from the working environment. The main source of all inorganic particles is most probably the environmental air as they form the major components of the particulate matters [35]. In addition, *p*H of the body fluids contributes to formation of calcite [36, 37] which is in agreement with the lower pH and a higher number of calcite in the EBC of the workers.

The question of the possible transport of nanoparticles to the urine is very important, as systemic effects of nanoparticles cannot be excluded since nanosized particles may reportedly cross the cellular membranes. The potential of TiO_2 nanoparticles to translocate in the body of experimental animals, including to the CNS, has been reported by several experimental studies. Oxidative stress occurred in the brains of mice exposed to TiO_2 nanoparticles of anatase and rutile crystal phases through nasal instillation, causing lipid peroxidation, protein oxidation and increased catalase activity [9, 10, 38].

Our data solely demonstrated that TiO_2 particles that are 100 nm or larger, identifiable by Raman microspectroscopy, were not eliminated in urine. However, the presence of TiO_2 nanoparticles in the urine samples still could not be excluded, since the Raman microspectroscopy method used in this study could only detect the particles/agglomerates that were 100 nm or larger in size. This is the main limitation of our study, because the presence of nanoparticles in the urine would be a very important finding. Additionally, this method of Raman microspectroscopy does not enable the quantitative analysis, as a finding of at least one TiO_2 crystal was classified as a positive finding.

Another limitation is the low number of exposed workers. In the nanoindustries, the number of exposed individuals during the production process is usually limited, and it is not feasible to have a high homogenous exposure in a large number of subjects. In addition, commonly, there is no motivation of the plants to participate in such studies. Therefore the studies performed *in vivo* with workers exposed to the aerosols containing engineered nanosized particles are important.

Among the advantages of this study is that there was detailed measurement of the aerosol levels at the workplaces. In addition, there was an increase of titanium in the EBC samples of workers at a RL4 compared to RL3, as well as RL3 to the controls.

In humans, the information about acute TiO_2 toxicity is very limited, and for nanoparticles, this information is currently unavailable. Epidemiological studies of adverse health effects induced by TiO_2 nanoparticles alone are lacking. The relatively short history of its production and use may be one of the explanations [2]. There are few cases of inhalational exposure in humans, with reports that a high concentration of TiO_2 may cause metal fume fever [39] or respiratory symptoms, accompanied by a reduction in pulmonary function [40].

In humans, severe symptoms in workers exposed to nanoparticles, including two deaths, were only described in one study, and in that study, the mechanism was not completely clear [41]. An inhalational exposure to nickel nanoparticles leading to the adult respiratory distress syndrome and death has been reported recently [42].

At this stage, risk characterisation of TiO_2 nanoparticles is hampered by the lack of relevant data on human exposure [35].

Due to its apparent simplicity and non-invasiveness, the EBC analysis is a promising technique, useful especially in subjects exposed to carcinogenic dusts. To our knowledge, it has not yet been used to study TiO_2 particles in highly exposed workers, and EBC collection appears to be an easy method to enable the detection of the particles by Raman microspectroscopy. The EBC analysis was shown to be useful in studies of volunteers with short-term exposures [43, 44], as well as in one recent large study of workers from 14 manufacturing plants, exposed to different types of engineered nanoparticles, which found a suppression of antioxidant enzymes [45].

5. Conclusions

EBC collection and analysis, combined with Raman microspectroscopy, enabled us to prove the presence of TiO_2 particles/agglomerates in the respiratory tract using non-invasive methods, in order to identify anatase and rutile crystal phases and measure the concentration of titanium in the samples. It was shown that during TiO_2 white pigment production, the workers of all parts of the production process, including the research part, were exposed to both anatase and rutile. These particles may persist in the lungs of exposed workers for at least several hours after the previous shift or several shifts. On the other hand, urine examination using Raman microspectroscopy produced negative results.

Conflict of interest

The authors claim no conflict of interests.

Acknowledgment

The authors would like to thank the Charles University in Prague project P 25/1LF/2, P28/1LF/6 and EU Project 'Material—Technical Research Base for the Diagnostics and Treatment of Environmentally-caused and Oncological Disorders and their Risks, in the General University Hospital in Prague' (reg. No. CZ.2.16/3.1.00/24.12) and the 'Operational Program Prague—Competitiveness' (CZ.2.16/3.1.00/22197), 'National Program of Sustainability' (NPU I (LO) MSMT- 34870/2013) and Czech Science Foundation project P503/12/G147 for their financial support.

References

[1] Karlsson H L, Gustafsson J, Cronholm P and Moller L 2009 Size-dependent toxicity of metal oxide particles-a comparison between nano- and micrometer size *Toxicol. Lett.* **188** 112–8

[2] Shi H B, Magaye R, Castranova V and Zhao J S 2013 Titanium dioxide nanoparticles: a review of current toxicological data *Part. Fibre Toxicol.* **10** 15

[3] Silva R M, TeeSy C, Franzi L, Weir A, Westerhoff P, Evans J E and Pinkerton K E 2013 Biological response to nano-scale titanium dioxide (TiO_2): role of particle dose, shape, and retention *J. Toxicol. Environ. Health* A **76** 953–72

[4] Warheit D B 2013 How to measure hazards/risks following exposures to nanoscale or pigment-grade titanium dioxide particles *Toxicol. Lett.* **220** 193–204

[5] Turci F, Peira E, Corazzari I, Fenoglio I, Trotta M and Fubini B 2013 Crystalline phase modulates the potency of nanometric TiO_2 to adhere to and perturb the stratum corneum of porcine skin under indoor light *Chem. Res. Toxicol.* **26** 1579–90

[6] Li B *et al* 2013 Molecular mechanisms of nanosized titanium dioxide-induced pulmonary injury in mice *PLoS One* **8** e55563

[7] Gurr J R, Wang A S, Chen C H and Jan K Y 2005 Ultrafine titanium dioxide particles in the absence of photoactivation can induce oxidative damage to human bronchial epithelial cells *Toxicology* **213** 66–73

[8] Chang X H, Zhang Y, Tang M and Wang B 2013 Health effects of exposure to nano-TiO_2: a meta-analysis of experimental studies *Nanoscale Res. Lett.* **8** 1–10

[9] Wang J *et al* 2008 Potential neurological lesion after nasal instillation of TiO_2 nanoparticles in the anatase and rutile crystal phases *Toxicol. Lett.* **183** 72–80

[10] Wang J *et al* 2007 Acute toxicity and biodistribution of different sized titanium dioxide particles in mice after oral administration *Toxicol. Lett.* **168** 176–85

[11] Horvath I *et al* 2005 Exhaled breath condensate: methodological recommendations and unresolved questions *Eur. Resp. J.* **26** 523–48

[12] Rosias P 2012 Methodological aspects of exhaled breath condensate collection and analysis *J. Breath Res.* **6** 027102

[13] Cherot-Kornobis N, Hulo S, de Broucker V, Hassoun S, Lepage N, Edme J L and Sobaszek A 2012 Induced sputum, exhaled NO, and breath condensate in occupational medicine *J. Occup. Environ. Med.* **54** 922–7

[14] Pelclova D, Fenclova Z, Kacer P, Kuzma M, Navratil T and Lebedova J 2008 Increased 8-isoprostane, a marker of oxidative stress in exhaled breath condensate in subjects with asbestos exposure *Ind. Health.* **46** 484–9

[15] Syslova K, Kacer P, Kuzma M, Pankracova A, Fenclova Z, Vlckova S, Lebedova J and Pelclova D 2010 LC-ESI-MS/MS method for oxidative stress multimarker screening in the exhaled breath condensate of asbestosis/silicosis patients *J. Breath Res.* **4** 017104

[16] Møller P, Jacobsen N R, Folkmann J K, Danielsen P H, Mikkelsen L, Hemmingsen J G, Vesterdal L K, Forchhammer L, Wallin H and Loft S 2010 Role of oxidative damage in toxicity of particulates *Free Radic. Res.* **44** 1–46

[17] Pelclova D, Fenclova Z, Syslova K, Vlckova S, Lebedova J, Pecha O, Belacek J, Navratil T, Kuzma M and Kacer P 2011 Oxidative stress markers in exhaled breath condensate in lung fibroses are not significantly affected by systemic diseases *Ind. Health* **49** 746–54

[18] Pelclova D *et al* 2012 Markers of oxidative stress are elevated in workers exposed to nanoparticles *Proc. of the 4th Int. Conf. on NANOCON; 2012* (*Brno, 23–25 October 2012*) (www.nanocon.eu/files/proceedings/04/reports/628.pdf)

[19] Falgayrac G, Cherot-Kornobis N, de Broucker V, Hulo S, Edme J L, Sobaszek A and Penel G 2011 Noninvasive molecular identification of particulate matter in lungs by Raman microspectrometry *J. Raman Spectrosc.* **42** 1484–7

[20] Hulo S, Cherot-Kornobis N, Edme J L, de Broucker V, Falgayrac G, Penel G, Legrand-Cattan K, Remy J and Sobaszek A 2013 Mica dust and pneumoconiosis example of a pure occupational exposure in a muscovite milling unit *J. Occup. Environ. Med.* **55** 1469–74

[21] IARC 2010 Carbon Black, Titanium Dioxide, and Talc *IARC Monogr. Eval. Carcinog. Risks Hum.* 93 (http://monographs.iarc.fr/ENG/Monographs/vol93/)

[22] Boffetta P *et al* 2004 Mortality among workers employed in the titanium dioxide production industry in Europe *Cancer Causes Control* **15** 697–706

[23] Dankovic D, Kuempel E, Geraci C, Gilbert S, Rice F, Schulte P, Smith R, Sofge C, Wheeler M and Zumwalde R 2011 Occupational exposure to titanium dioxide Current Intelligence Bulletin 63. 63 (Department of Health and Human Services, Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health) p 1–140

[24] EASHW 2014 Priorities for occupational safety and health research in Europe for the years 2013–2020 (Luxembourg: Publications Office of the European Union; European Agency for Safety and Health at Work) (https://osha.europa.eu/en/publications/reports/summarypriorities-for-osh-research-in-eu-for-2013–20)

[25] Paik S Y, Zalk D M and Swuste P 2008 Application of a pilot control banding tool for risk level assessment and control of nanoparticle exposures *Ann. Occup. Hyg.* **52** 419–28

[26] Effros R M, Biller J, Foss B, Hoagland K, Dunning M B, Castillo D, Bosbous M, Sun F and Shaker R 2003 A simple method for estimating respiratory solute dilution in exhaled breath condensates *Am. J. Respir. Crit. Care Med.* **168** 1500–5

[27] Orazem E L 2007 *Raman Spectroscopy on the Nanoscale: An Investigation of Silver Triangle Arrays, Polymer Laminates and Gold-Loaded Mesoporous Titania Films* (Ann Arbor, MI: ProQuest)

[28] Ohsaka T, Izumi F and Fujiki Y 2005 Raman spectrum of anatase, TiO_2 *J. Raman Spectrosc.* **7** 321–4

[29] Kelly S, Pollak F H and Tomkiewicz M 1997 Raman spectroscopy as a morphological probe for TiO_2 aerogels *J. Phys. Chem.* B **101** 2730–4

[30] Miyagi T, Kamei M, Mitsuhashi T, Ishigaki T and Yamazaki A 2004 Charge separation at the rutile/anatase interface: a dominant factor of photocatalytic activity *Chem. Phys. Lett.* **390** 399–402

[31] Kostikas K, Papatheodorou G, Ganas K, Psathakis K, Panagou P and Loukides S 2002 *p*H in expired breath condensate of patients with inflammatory airway diseases *Am. J. Respir. Crit. Care Med.* **165** 1364–70

[32] Bakand S, Hayes A and Dechsakulthorn F 2012 Nanoparticles: a review of particle toxicology following inhalation exposure *Inhal. Toxicol.* **24** 125–35

[33] Kwon S, Yang Y S, Yang H S, Lee J, Kang M S, Lee B S, Lee K and Song CW 2012 Nasal and pulmonary toxicity of titanium dioxide nanoparticles in rats *Toxicol. Res.* **28** 217–24

[34] Kreyling W G, Semmler-Behnke M, Takenaka S and Möller W 2013 Differences in the biokinetics of inhaled nano- versus micrometer-sized particles *Acc. Chem. Res.* **46** 714–22

[35] Harrison R M, Jones A M and Lawrence R G 2004 Major component composition of PM10 and PM2.5 from roadside and urban background sites *Atmos. Environ.* **38** 4531–8

[36] Ruiz-Agudo E, Putnis C V, Rodriguez-Navarro C and Putnis A 2011 Effect of pH on calcite growth at constant view the MathML source a_{Ca2+}/a_{CO32-} ratio and supersaturation *Geochim. Cosmochim. Acta* **75** 284–96

[37] Oyane A, Onuma K, Ito A, Kim H M, Kokubo T and Nakamura T 2003 Formation and growth of clusters in conventional and new kinds of simulated body fluids *J. Biomed. Mater. Res.* A **64** 339–48

[38] Hu R, Gong X, Duan Y, Li N, Che Y, Cui Y, Zhou M, Liu C, Wang H and Hong F 2010 Neurotoxicological effects and the impairment of spatial recognition memory in mice caused by exposure to TiO_2 nanoparticles *Biomaterials* **31** 8043–50

[39] Otani N, Ishimatsu S and Mochizuki T 2008 Acute group poisoning by titanium dioxide: inhalation exposure

may cause metal fume fever *Am. J. Emerg. Med.* **26** 608–11
[40] Garabrant D H, Fine L J, Oliver C, Bernstein L and Peters J M 1987 Abnormalities of pulmonary function and pleural disease among titanium metal production workers *Scand. J. Work Environ. Health* **13** 47–51
[41] Song Y and Tang S 2011 Nanoexposure, unusual diseases, and new health and safety concerns *Sci. World J.* **11** 1821–8
[42] Phillips J I, Green F Y, Davies J C and Murray J 2010 Pulmonary and systemic toxicity following exposure to nickel nanoparticles *Am. J. Ind. Med.* **53** 763–7
[43] Svedahl S R, Svendsen K, Tufvesson E, Romundstad P R, Sjaastad A K, Qvenild T and Hilt B 2013 Inflammatory markers in blood and exhaled air after short-term exposure to cooking fumes *Ann. Occup. Hyg.* **57** 230–9
[44] Sauvain J J, Sanchez Sandoval Hohl M, Wild P, Pralong J A and Riediker M 2014 Exhaled breath condensate as a matrix for combustion-based nanoparticle exposure and health effect evaluation *J. Aerosol Med. Pulm. Drug Deliv.* **27** 449–58
[45] Liou S H *et al* 2012 Epidemiological study of health hazards among workers handling engineered nanomaterials *J. Nanopart. Res.* **14** 878