Research Article

Evaluation of Phototoxic and Cytotoxic Potential of TiO_2 Nanosheets in a 3D Reconstructed Human Skin Model

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Abstract

Despite a continuous increase in commercial products containing nanoparticles, only few materials are currently used in such large amounts, forms and sizes as titanium dioxide – TiO_2. Besides their use in cosmetics, the food industry and biomedicine, TiO_2 nanoparticles (NPs) are also used as highly efficient photocatalysts due to their unique ability to convert complex organic materials to carbon dioxide, water and simple mineral acids via complex radical and electron transfer reactions. We assessed the potential dermal effect (phototoxicity and skin toxicity) of TiO_2 nanosheets (TIG-800) synthesized from the lyophilized aqueous colloids of peroxo-polytitanic acid at high temperatures. The nanoparticles were examined in the reconstructed human skin model EpiDerm™ in a test pre-validated by ECVAM and adopted into the ICH S10 guidelines for the preclinical photo-safety assessment of drugs. For comparison, two further commercial samples of nanocrystalline TiO_2, Aeroxide P25 and Eusolex T-2000, and six benchmark materials from pre-validation studies were tested. None of the TiO_2 NPs tested in the study caused acute phototoxicity or cytotoxicity in the reconstructed 3D tissues up to the highest concentration tested. The prediction of photo-irritation potency for the benchmark chemicals was comparable to previous studies. For some of the tested materials, we identified reasons for false negative results or variability in previously published datasets by improving dosing, conditions of irradiation, and choice of suitable solvents. The method proved its suitability for photo-irritation assessment of topically applied materials.

1 Introduction

Titanium dioxide (TiO_2) has been widely used in a variety of industrial applications for decades and is thus considered an inert and safe material. However, with the development of nanotechnologies and reported health risks caused by some nanoparticles (NPs), TiO_2 in nano-form is now under toxicological scrutiny. Mechanistic toxicological studies have shown that TiO_2 nanoparticles predominantly cause adverse effects via induction of oxidative stress, resulting in cell damage, genotoxicity, inflammation and immune response. The extent and type of damage strongly depends on the physical and chemical characteristics of the TiO_2 nanoparticles, which govern their bioavailability and reactivity (Skocaj et al., 2011).

Studies on dermal exposure, conducted due to the wide use of TiO_2 in cosmetics, as color additive and sunlight blocker, have shown repeatedly that TiO_2, even in nano-form, has only minimal potential to penetrate healthy human skin and reach the bloodstream. It has been reported that TiO_2 nanoparticles may be able to penetrate the skin surface through hair follicles or pores, but no details were given on the fate of such particles (Danish EPA, 2015).

The pigment form of TiO_2 is certified by the US Food and Drug Administration (21 CFR 73.2575) and in the EU as a color additive (E 171); while the non-pigment form TiO_2 is certified for safe use by the United States Pharmacopeia, and in the EU it is listed in Annex VI of the Cosmetics Regulation 1223/2009. Under normal, foreseeable conditions of use, TiO_2 is considered a safe cosmetic ingredient, even in the nano-form.

A different situation may arise in occupational exposure to TiO_2, where workers may be exposed to airborne particles of various sizes in excessive amounts. Workers who are not protect-

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Tab. 1: Characteristics of three TiO_2 nanoparticles used in the study

Chemical	CAS	Purity	Supplier	Dispersed in	A_{max} (nm)
TiO_2 – TIG-800	13463-67-7	n/a	Czech Academy of Sciences	DI H_2O	369
TiO_2 – Aeroxid P25	13463-67-7	≥ 99.5%	Evonik	DI H_2O	364
TiO_2 – Eusolex T-2000	13463-67-7	n/a	Merck KGaA	DI H_2O	364

A_{max}, absorption maximum (spectrophotometry/information from the supplier); DI, deionized

Tab. 2: Characterization of reference chemicals

Chemical (abbreviation)	CAS	Purity	Supplier	Solvent	A_{max} (nm)	PT/NPT	Use
Chlorpromazine hydrochloride (CPZ)	69-09-0	≥ 95%	Sigma-Aldrich	H_2O	310	PT	Pharmaceutical, positive control
Anthracene (AN)	120-12-7	≥ 95%	Sigma-Aldrich	EtOH or AC:OO (4:1)	356	PT*	Pharmaceutical
Bergamot oil (BO)	8007-75-8	≥ 35%	Sigma-Aldrich	SSO	334	PT	Cosmetics, food industry
Bergamot oil (BO) – Kosher	8007-75-9	n/a	Sigma-Aldrich	SSO	326	PT	Cosmetics, food industry
Cinnamaldehyde (CA)	104-55-2	≥ 95%	Sigma-Aldrich	SSO	336	NPT	Cosmetics, food industry
4-Aminobenzoic acid (PABA)	150-13-0	≥ 99%	Sigma-Aldrich	SSO	325	NPT	Cosmetics

* material with tendency to false-negative results; A_{max}, absorption maximum; SSO, sesame seed oil; H_2O, ultra-pure water; EtOH, ethanol; AC:OO, mixture of acetone and olive oil; PT, phototoxic; NPT, non-phototoxic

ed by appropriate clothing and respirators may achieve topical and systemic exposure to the TiO_2 NPs that could lead to chronic health effects.

In the current study, taking into account the occupational exposure scenario, we evaluated the dermal effects of TiO_2 nanosheets (TIG-800) synthesized from lyophilized aqueous colloids of peroxo-polytitanic acid at a temperature of 800°C. Such TiO_2 nanosheets possess high photocatalytic activity (Šubrt et al., 2014; Pližingrová et al., 2017) and thus may be suitable for use in environmental purification processes. Acute phototoxicity and cytotoxicity studies were conducted in the 3D reconstructed human skin model EpiDerm™, a validated and regulatory accepted tissue model for dermal toxicity testing. Within different protocols, the model has been implemented in regulations for testing of chemicals (OECD TG 431 and 439), regulations for pre-clinical phototoxicity testing of pharmaceuticals (EMA ICH S10), and also in standards for medical device testing (ISO standard 10993-23).

To ensure that the method is well-established in our laboratory and provides relevant information on phototoxicity and cytotoxicity, we tested two other forms of TiO_2 NPs available from commercial sources (Tab. 1) along with the TIG-800 NPs as well as a set of six known phototoxic or non-phototoxic reference chemicals with differing bioavailability and dermal toxicity effects (Tab. 2).

2 Materials and methods

Test and reference chemicals

TIG-800 were prepared from the lyophilized aqueous colloids of peroxo-polytitanic acid at a temperature of 800°C as described (Šubrt et al., 2014; Pližingrová et al., 2017). The peroxo-polytitanic acid foams annealed at temperatures above 500°C to provide anatase in the form of thin leaves consisting of intergrown nanocrystalline anatase particles, which transform at temperatures above 850°C to rutile. The detailed description of the syn-

Abbreviations
3D, three dimensional; AC:OO, mixture of acetone and olive oil; A_{max}, absorption maximum; AN, anthracene; BO, bergamot oil; CA, cinnamaldehyde; CAS, chemical abstracts service; CPZ, chlorpromazine hydrochloride; DI, deionized water; DPBS, Dulbecco's phosphate-buffered saline solution; ECVAM, European Centre for Validation of Alternative Methods; EMA, European Medicines Agency; EtOH, ethanol; EU, European Union; FDA, Food and Drug Administration; H_2O, ultra-pure water; H3D PT, phototoxicity test on reconstructed human 3D tissue EpiDerm; ICH, International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NP, nanoparticle; NPT, non-phototoxic; OECD, Organisation for Economic Co-operation and Development; PABA, 4-aminobenzoic acid; PT, phototoxic; SCC, standard culture conditions; SCCS, Scientific Committee on Consumer Safety; SSO, sesame seed oil; US, United States; UV, ultraviolet

thetic procedure and the dependence of TiO_2 phase composition on the annealing temperature of the lyophilized peroxo-polytitanic acid gel is described in detail elsewhere (Šubrt et al., 2014). SEM micrographs evidence that the TIG-800, annealed at 800°C, represents very thin foils with sizes in the range of mm up to cm, possessing a thickness below 100 nm, which are composed of closely packed crystals of anatase with the crystallite size of about 50 nm (Pližingrová et al., 2015, 2017). The TIG-800 nanosheets have a specific surface area of 26 $m^2 g^{-1}$, low apparent density, high porosity and high sorption ability (Pližingrová et al., 2017). These properties enable a broad application of this form of 2D-TiO_2 in industry, especially in environmental purification processes (Šubrt et al., 2014).

AEROXIDE® TiO_2 P25[1] are chemically and thermally stable agglomerates of titanium dioxide nanoparticles with a specific surface area in the range of 35-65 $m^2 g^{-1}$ and an average particle size of 21 ±5 nm. The material represents a unique combination of anatase (70%) and rutile (20%) crystal structures with a small contribution of the amorphous phase. AEROXIDE® TiO_2 P25 has excellent catalytic and photocatalytic activity. It is used for catalytic processes of substrates with high thermal stability and as a component of self-cleaning construction materials such as concrete, rock or mineral plaster, in the production of solar cells, or as a UV-absorbing component of inorganic substances (Ohtani et al., 2010). According to the information given by the producer, Aeroxide P25 contains 99.5% TiO_2, with small amounts of Al_2O_3, SiO_2 and Fe_2O_3, and is without any surface modification.

Eusolex® T-2000[1] (inorganic UV filter) is a fine white powder of titanium dioxide with a modified surface (alumina and simethicone (poly(dimethylsiloxane)) with added silica gel). It consists of 10-15 nm particles and 100 nm needle aggregates. Eusolex T-2000 represents the rutile allotrope of TiO_2 and possesses an amphiphilic character. Therefore, it can be used in the water or oil phase of cosmetic products. It is used as a UVA-filter (blocker) in cosmetics, particularly in UV-protective creams for children, infants and persons with very pale or sun-sensitive skin. It is also a component of daily skincare products and make-up foundation creams (PROSPECTOR®, 2016). Eusolex T-2000 exhibits a specific surface area of 83.2 $m^2 g^{-1}$ (Strobel et al., 2014).

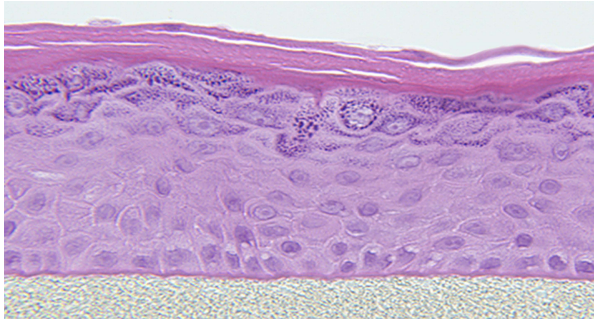

Fig. 1: Reconstructed human skin model EpiDerm™
(Courtesy of MatTek Corporation)

The TiO_2 NPs were purchased as powders. The stock dispersions of TiO_2 NP were prepared as 1% w/v dispersions in deionized (DI) water. In one case, we also tested 3.16% prepared from the 10% stock. Stock dispersions were thoroughly vortexed and then sonicated in an ultrasonic bath set to 80 W for 15 min. Thereafter, following the instructions of the SOP, we diluted the 1% stock solution dispersions to 0.316%, 0.1%, 0.0316%, 0.01% and 0.00316%. The concentrations were calculated using the following formulae:

$$\text{w/v (\%)} = \frac{\text{mass of solute (g)}}{\text{volume of solution (mL)}} \times 100$$

$$\text{v/v (\%)} = \frac{\text{volume of solute (mL)}}{\text{volume of solution (mL)}} \times 100$$

Six reference chemicals with known phototoxic and skin irritation responses, summarized in Table 2, were used as positive controls. These materials had been used in the development and pre-validation study of the reconstructed human 3D tissue model EpiDerm™ (further abbreviated as H3D PT) conducted by Liebsch et al. (1997, 1999).

Test system – Reconstructed human skin model

The reconstructed human 3D tissue model EpiDerm™ (H3D PT) (MatTek, Ashland, USA and MatTek IVLSL, Bratislava, Slovakia, EU) (Fig. 1) consists of normal, human-derived epidermal keratinocytes cultured to form a multilayered, highly differentiated model of the human epidermis. It consists of organized basal, spinous and granular layers, and a multilayered *stratum corneum* containing intercellular lamellar lipid layers arranged in patterns analogous to those found *in vivo* (Cannon et al., 1994). The epidermal cells were taken from healthy volunteers who were negative for HIV and hepatitis. EpiDerm™ tissues (surface 0.63 cm^2) are cultured on cell culture inserts and shipped as kits containing 24 tissues on shipping agarose together with culture medium and 6-well plates. In addition, an MTT kit (containing MTT concentrate, diluent, extractant), Dulbecco's phosphate-buffered saline solution (DPBS, without calcium and magnesium) and a 24-well plate are provided in the kit.

The EpiDerm™ model and testing kit are manufactured according to defined quality assurance procedures compliant with GMP and the ISO 9001:2015 process. All media and EpiDerm™ tissues are serum-free. All biological components of the epidermis and the culture medium are tested by the manufacturer for viral, bacterial, fungal and mycoplasma contamination. Barrier properties of each manufactured tissue lot are controlled by the manufacturer. Tissues used in the current study were obtained from MatTek In Vitro Life Science Laboratories, Bratislava, Slovakia.

[1] https://products-re.evonik.com/www2/uploads/productfinder/AEROXIDE-TiO2-P-25-EN.pdf
[2] https://www.ulprospector.com/en/asia/PersonalCare/Detail/1193/34422/Eusolex-T-2000

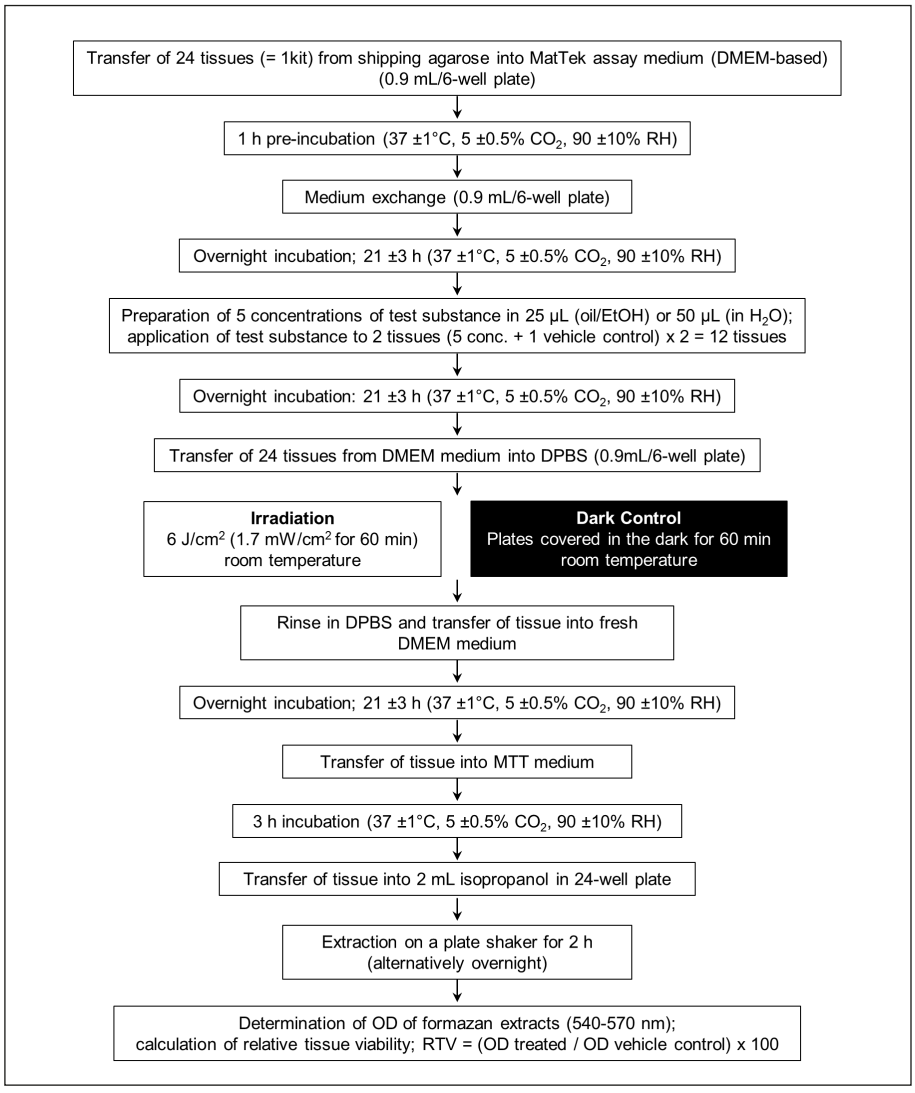

Fig. 2: Scheme of the H3D PT protocol
(according to Liebsch et al., 1997 with modifications)

EpiDerm™ phototoxicity test (H3D PT)

H3D PT was performed according to SOP ZEBET "*Phototoxicity Protocol For Use with EpiDerm™ Model (EPI-200)*"[3] (Liebsch et al., 1997) with minor modifications that reflect long-term experience gained with this test over almost 20 years (for details see Kandárová and Liebsch, 2017).

EpiDerm tissues were transferred from shipping agar into the 6-well plates containing 0.9 mL assay medium per well and were pre-incubated in an incubator for 1 h at standard culture conditions (SCC = 37 ±1°C, 90 ±10% RH, 5 ±0.5% CO_2) to allow release of metabolites accumulated during storage on agar. After 1 h, tissue models were transferred into 6-well plates pre-filled with fresh assay medium and pre-incubated overnight at SCC.

On the first day of the experiment, the concentration range for each tested chemical was prepared. The following solvents were used to dissolve the test materials: ultra-pure water, ethanol [64-17-5] (Sigma-Aldrich, purity ≥ 99.8%), sesame seed oil (SSO) [8008-74-0] (Sigma Aldrich, pharm. grade), and acetone: olive oil (AC:OO) mixture (4:1, v/v); acetone [67-64-1] (Sigma-Aldrich) and olive oil [8008-74-0] (Sigma-Aldrich, Phar.grade). 50 µL aqueous solutions or dispersions or 25 µL ethanol or SSO solutions were applied to the apical surface of the tissue. Four tissues were used per concentration tested. Tissues were incubated with the test material for 21 ±3 h at SCC.

On the next day, half of the tissues were transferred into 6-well plates containing 0.9 mL DPBS and were irradiated using a Dr. Hönle 500 solar simulator (Dr. Hönle AG UV Technology, Germany) equipped with a mercury-halide bulb and an H1-filter. The device was mounted on a stable holder allowing adjustment of the exposure distance to 60 ±5 cm to achieve irradiance of 1.7 ±0.2 mW/cm² measured through the lid of a cell culture plate using a calibrated UVX radiometer (UVP Ltd., UK). Tissue sam-

[3] https://www.mattek.com/wp-content/uploads/Phototoxicity-Protocol.pdf

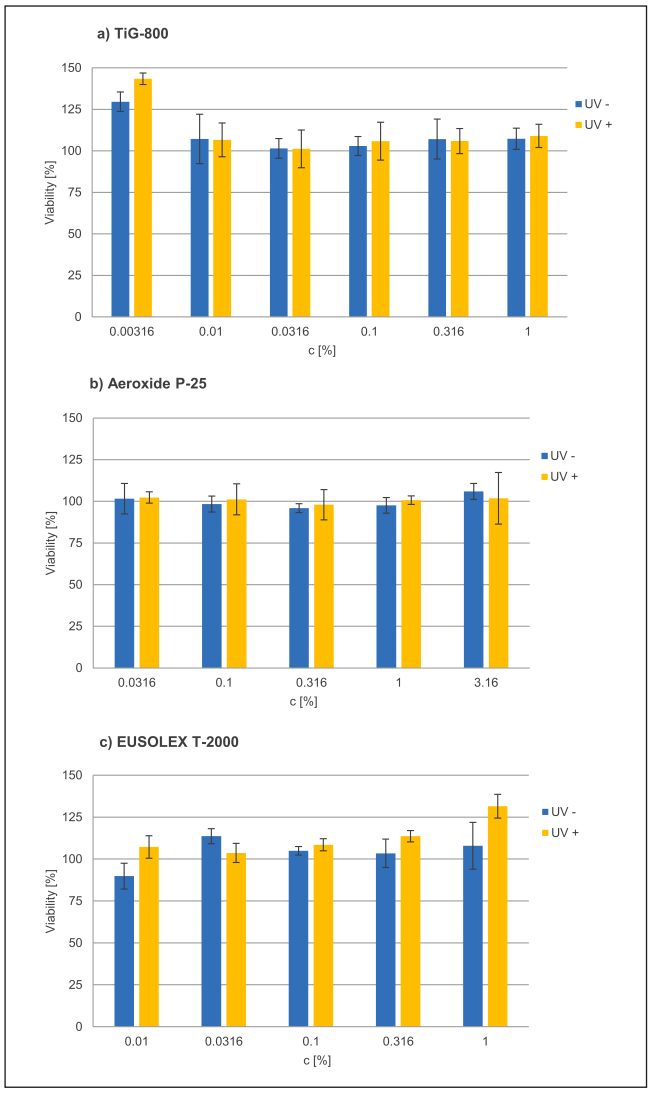

Fig. 3: Dose-response of the H3D PT to TiO_2 NPs in the presence and absence of UVA/VIS light

a) TIG-800, b) Aeroxide P25, and c) EUSOLEX T-2000. Dispersions were prepared in ultrapure H_2O and applied in a volume of 50 μL (N = 2). The highest concentration of TiO_2 NPs tested was in the range of 1-3.16% to avoid UV-protection of the tissue by the formation of a continuous layer of TiO_2 on the tissue surface.

ples were irradiated for 60 min; the resulting UVA dose was 6 J/cm^2. Non-irradiated controls were placed into 6-well plates pre-filled with DPBS and covered with aluminum foil or stored in the dark at room temperature until irradiation of the other tissue samples was completed. After irradiation, tissues were washed in DPBS and incubated at SCC for 21 ±3 h.

On the third day, tissue viability was determined in an MTT assay. Inserts were transferred from the 6-well plates into 24-well plates pre-filled with 0.3 mL MTT (1 mg/mL) and incubated at SCC for 3 h. Afterwards, inserts were transferred and submerged into 2 mL isopropanol in 24-well plates, sealed with parafilm, covered with aluminum foil and placed on a plate shaker for at least 2 h (alternatively, extracted overnight in the dark). Inserts containing skin tissue treated with TiO_2 NPs were placed into 2 mL isopropanol in 6-well plates and extracted only through the basolateral side to avoid NPs trapped on the tissue surface entering the extraction solution. After the extraction was completed, inserts were discarded and the extract was homogenized by pipetting up and down at least three times. 200 µL extract per samples was transferred into a 96-well flat-bottom microtiter plate. Isopropanol was used as a blank. Optical density was measured using a 96-well plate spectrophotometer equipped with a 540-570 nm filter. No reference filter was used.

Using an automated spreadsheet, the difference in viability between tissue duplicates was calculated. If the difference in viability between irradiated and non-irradiated samples was ≥ 30% at one or more concentrations, the tested chemical was classified as phototoxic. The irritation potential of the test material was assessed in the non-irradiated samples. If the tissue viability decreased below 50%, this concentration of test material was considered likely irritating on human skin. The schematic overview of the test is shown in Figure 2.

3 Results

3.1 Phototoxicity and cytotoxicity effects of the studied TiO_2 nanoparticle samples

The results obtained with the three TiO_2 NPs are summarized in Table 3 and Figure 3a-c. None of the TiO_2 NPs tested in the study caused phototoxicity or cytotoxicity up to the highest concentration tested.

3.2 Phototoxic and cytotoxic effect of reference chemicals

Six reference chemicals, four phototoxic (PT) and two non-phototoxic (NPT), chosen from OECD TG 432 (3T3 NRU PT) and publications related to the pre-validation of H3D PT, were used to demonstrate that the method was performed correctly and the minor modifications introduced, reflecting our experience with this method, did not affect the correct prediction of phototoxicity and phototoxic potency. The results obtained for the reference chemicals are shown in Table 4 and Figure 4.

A phototoxic effect was correctly predicted for all four phototoxic chemicals, namely chlorpromazine hydrochloride, bergamot oil, bergamot oil - kosher and anthracene. The two non-phototoxic chemicals, cinnamaldehyde and 4-aminobenzoic acid, were predicted correctly by the H3D PT as non-phototoxic.

Chlorpromazine hydrochloride (CPZ) is used as a positive control in the H3D PT as well as in OECD TG 432. CPZ was tested in three independent experiments in concentrations ranging from 0.001% to 0.1% (Fig. 4a). The decrease in the viability of irradiated tissues occurred within the range of 0.005% to 0.01%. The first cytotoxic concentration in the 3D model was 0.05%. These results reflect the results obtained by the test method developer (Liebsch et al., 1997, 1999).

The phototoxic potential of anthracene (AN) was examined in the concentration range of 0.01-1%. Despite some data reported earlier on false-negative results of anthracene in the H3D PT

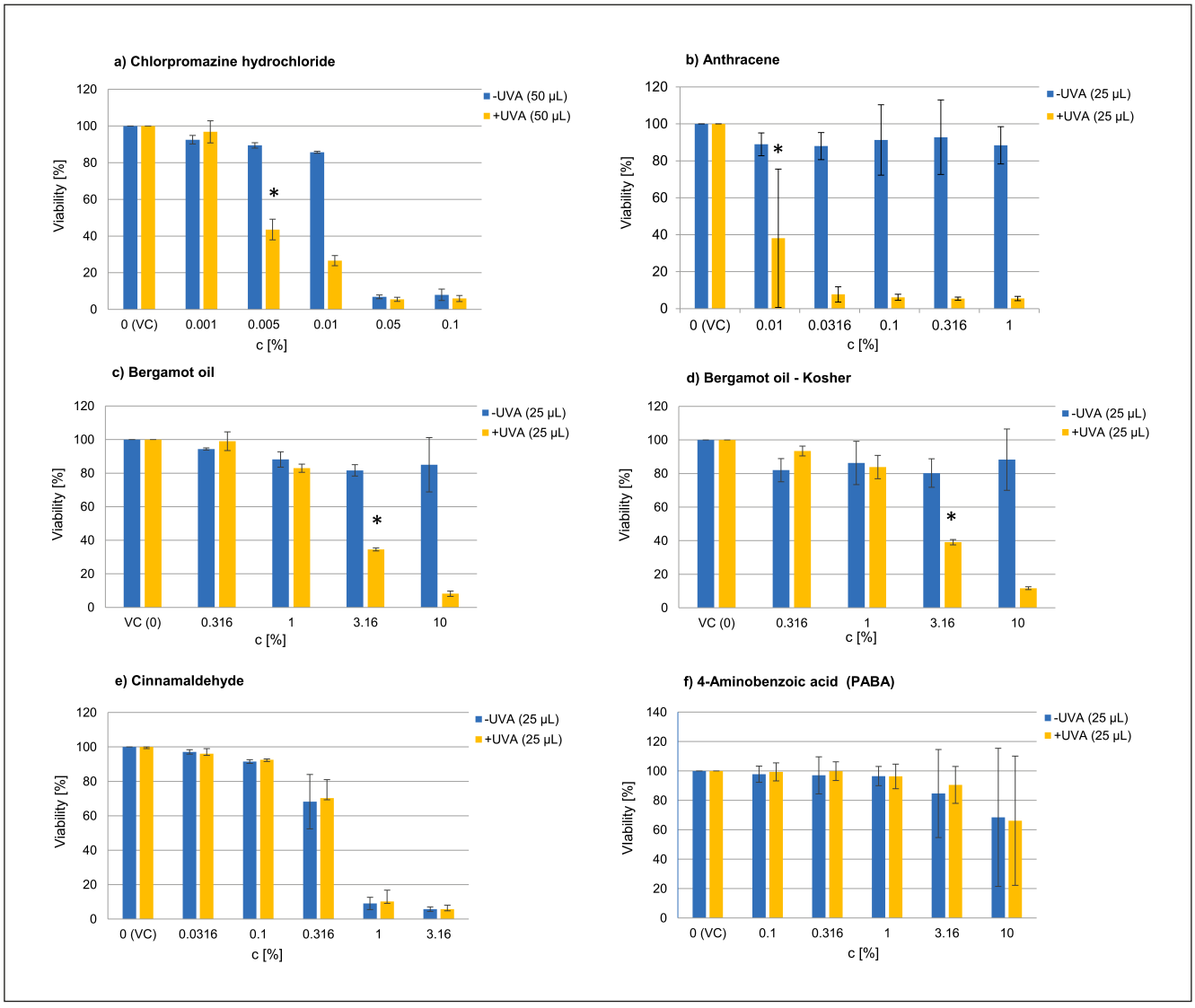

Fig. 4: Dose-response of the H3D PT to reference chemicals

Response to a) chlorpromazine hydrochloride, b) anthracene, c) bergamot oil, d) bergamot oil - kosher, e) cinnamaldehyde, f) 4-amino-benzoic acid (PABA) in the presence (yellow) and absence (blue) of UVA/VIS light

* The first concentration of the test substance for which a phototoxic effect was observed. VC-vehicle control

(Liebsch et al., 1997), in the current study, we found phototoxic potency already at the lowest concentration tested, i.e., 0.01% in ethanol (Fig. 4b) as well as in alternative solvent AC:OO (4:1) (Fig. 5).

Two types of bergamot oil (BO) from Sigma (part No. B-4383 and W-215309 - Kosher) were tested in two independent experiments. Samples were studied at a concentration range of 0.1-10%, i.e., concentrations that are typical for use in the food and fragrance industries. A significant decrease in tissue viability in the concentration range between 1-3.16% was observed for both types of bergamot oil after irradiation (Fig. 4c,d). There was no difference between the cytotoxicity and phototoxicity of the two samples. The International Fragrance Association recommends using bergamot oil at a maximum concentration of 0.4%. Our study confirms that the phototoxic effect does not occur at concentrations close to the highest recommended dose.

Cinnamaldehyde (CA) was tested at a concentration range of 0.0316-3.16% using SSO as the solvent. Figure 4e shows an almost identical decrease in tissue viability at concentrations of 0.316% and 1% in the irradiated as well as non-irradiated tissues. These results indicate that cinnamaldehyde is not phototoxic, but it has a cytotoxic effect at these concentrations, which indicates that skin irritation may be expected in sensitive human volunteers at these concentrations.

4-Aminobenzoic acid (PABA) was tested in the range of 0.1-10%, reflecting concentrations used in cosmetics outside the EU. The use of PABA as a cosmetic UV filter has been discontinued in the EU due to toxicological concerns. In the current test, it was

Tab. 3: Viability of H3D PT after treatment with TiO_2 NPs, with and without irradiation, and resulting prediction

Test chemical	Conc. (%) (w/v)	EpiDerm™ viability -UVA (%)	D (%)	EpiDerm™ viability +UVA (%)	D (%)	Δ (%)	Prediction
TIG-800	0.00316	129.6	11.8	143.4	7.0	< 30	Non-phototoxic and non-cytotoxic up to the highest conc. tested
	0.01	107.2	29.8	106.6	20.4		
	0.0316	101.5	11.8	101.2	22.8		
	0.1	102.9	11.4	105.8	22.8		
	0.316	107.1	24.0	105.9	15.0		
	1.0	107.3	12.8	109.0	14.0		
Aeroxide P25	0.0316	101.6	18.2	102.3	6.8	< 30	Non-phototoxic and non-cytotoxic up to the highest conc. tested
	0.1	98.4	9.6	101.2	18.6		
	0.316	95.9	5.4	98.0	18.2		
	1.0	97.6	9.4	100.7	5.2		
	3.16	106.0	9.6	101.8	31.0		
Eusolex T-2000	0.01	89.8	15.4	107.2	13.4	< 30	Non-phototoxic and non-cytotoxic up to the highest conc. tested
	0.0316	113.6	9.0	103.6	11.4		
	0.1	104.9	5.0	108.5	7.2		
	0.316	103.4	17.0	113.6	6.8		
	1.0	107.9	28.0	131.5	14.2		

D, difference between 2 tissues treated with the NPs; Δ, difference between irradiated and non-irradiated tissue. If Δ in any of the tested concentrations > 30%, the chemical is classified as phototoxic.

Tab. 4: Viability of H3D PT after treatment with benchmark chemicals, with and without irradiation, and resulting prediction

Test chemical	Conc. (%) (w/v)	EpiDerm™ viability -UVA (%)	D (%)	EpiDerm™ viability +UVA (%)	D (%)	Δ (%)	Prediction
Chlorpromazine hydrochloride (PT)	0.001	92.4	2.3	99.2	6.1	≥ 30	Phototoxic, cytotoxic at 0.05%
	0.005*	88.8	1.8	43.6	5.6		
	0.01	84.4	2.3	25.1	2.8		
	0.05**	33.0	45.3	5.5	1.1		
	0.1	10.2	4.9	5.3	1.3		
Bergamot oil (PT)	0.316	94.4	1.4	99.1	11.2	≥ 30	Phototoxic, non-cytotoxic up to 10%
	1	88.1	9.2	83.0	5.0		
	3.16*	81.6	6.8	34.5	1.8		
	10	85.0	32.4	8.1	3.2		
Bergamot oil – Kosher (PT)	0.316	82.0	13.8	93.4	5.8	≥ 30	Phototoxic, non-cytotoxic up to 10%
	1	86.3	25.8	83.9	13.8		
	3.16*	80.3	17.0	39.1	3.2		
	10	88.2	36.6	11.7	1.6		
Anthracene (PT)	0.01*	89.0	6.2	38.1	37.5	≥ 30	Phototoxic, non-cytotoxic up to 1%
	0.0316	88.0	7.4	7.7	4.1		
	0.01	91.3	19.1	6.1	1.7		
	0.316	92.8	20.2	5.4	0.8		
	1	88.4	10.1	5.5	1.2		
Cinnamaldehyde	0.0316	97.1	2.6	96.0	6.0	< 30	Non-phototoxic, (NPT) cytotoxic at 1%
	0,1	91.5	2.2	92.6	0.8		
	0.316	68.2	31.6	70.2	21.4		
	1**	9.0	7.2	10.2	13.2		
	3.16	5.7	2.6	5.8	4.6		
4-Aminobenzoic acid (NPT)	0.1	97.8	5.5	99.4	6.2	< 30	Non-phototoxic, non-cytotoxic up to 10%, high variability
	0.316	97.0	12.7	99.8	6.4		
	1	96.5	6.5	96.3	8.4		
	3.16	84.7	30.0	90.5	12.5		
	10	68.4	47.0	66.2	43.9		

* first phototoxic concentration; ** first significant cytotoxic concentration (viability below 50% in the non-irradiated part of the experiment); NPT, non-phototoxic; PT, phototoxic

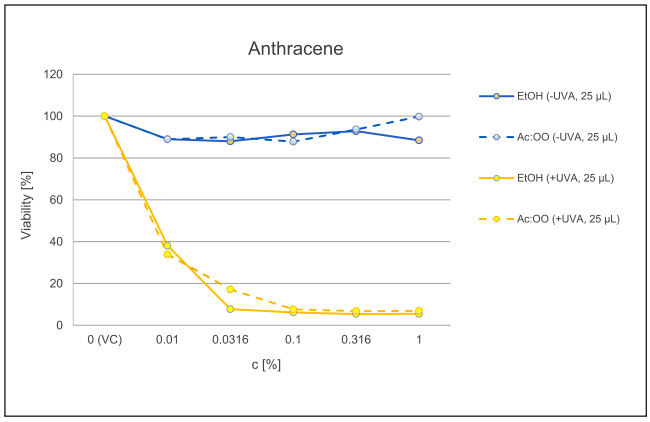

Fig. 5: Dose-response of the H3D PT to anthracene dissolved in ethanol or acetone-olive oil (4:1)

classified as a non-phototoxic substance with cytotoxic effects starting at 3.16% and substantial effects at 10%.

We conclude that the H3D PT provided expected phototoxicity and cytotoxicity responses to the reference materials and thus the test was properly calibrated for the prediction of the phototoxic effect of new materials.

4 Discussion

More and more commercial products contain nanoparticles, however, only few materials are currently used in such large amounts, forms and sizes as TiO_2. TiO_2 is extensively used as a pigment, a thickener and a UV absorber in cosmetic and skin-care products. In the field of nanomedicine, TiO_2 NPs allow osseointegration of artificial medical implants and bone (Gupta and Xie, 2018), they are used in photodynamic therapy to treat acne vulgaris, recurrent genital condylomas, atopic dermatitis, hyperpigmented lesions and other diseases, mainly due to their antibacterial effects (Shi et al., 2013). TiO_2 nanoparticles are also used as highly efficient photocatalysts due to their ability to convert organic compounds such as alcohols, carboxylic acid, phenolic derivatives, and chlorinated aromatics into carbon dioxide, water and simple mineral acids (Rezaei and Mosaddeghi, 2009).

TiO_2, in micro- or nanoform can be safe or unsafe, depending on multiple factors, including the form and size of the NPs and the type of exposure. TiO_2 is known in several modifications – in addition to rutile, anatase, akaogiite, and brookite, three metastable phases can be produced synthetically (monoclinic, tetragonal and orthorhombic), and five high-pressure forms also exist (Marshall, 2018). The size of TiO_2 NPs assessed for toxicological concerns starts at 4 nm.

In general, it is thought that TiO_2 NPs only penetrate healthy, intact human skin minimally and that the penetration route into the dermis is predominantly via hair channels and sweat glands (Danish EPA, 2015). Health risks are however recognized for oral intake (in the form of a food additive, where TiO_2 serves as a whitener) and via inhalation mainly due to occupational exposure (Danish EPA, 2015; NIOSH, 2011). With increasing knowledge of TiO_2 NPs toxicity, some EU countries are considering to ban the use of TiO_2 NPs as a food additive (E171) since it has purely aesthetic value, exhibits no nutritional value and may, on the other hand, pose serious risks to human health especially upon long-term use.

Here, we studied TiO_2 anatase nanosheets (TIG-800). Due to organization of the particles into the lamellar shape, the product has low apparent density, high porosity and high sorption ability, and as such, it presents an ideal material for photocatalytic reactions. Since occupational safety was of concern for the planned production of experimental batches, we investigated possible acute dermal irritation and phototoxicity of the TIG-800 NPs.

OECD TG 432, although validated and regulatory accepted for *in vitro* phototoxicity testing, is unfortunately not relevant for testing dermal exposure to TiO_2, as it is based on a submerged permanent cell line of mouse fibroblasts with no barrier properties. This system would provide false-positive results due to its lack of skin barrier properties and also due to the aqueous environment, which promotes radical oxygen reactions. It has been shown in previous studies that TiO_2 NPs accumulate in the cytoplasm of the mouse fibroblasts and cause phototoxicity by ROS generation (Fig. 6) (Kandárová, 2006). Such a situation, however, does not reflect the dermal route of exposure, since human skin is protected against xenobiotic penetration by several layers of stratum corneum (10-30 µm thick) and lipid layers.

A phototoxicity test on an *in vitro* 3D reconstructed human skin model was therefore considered a better approach, especially due to the similarity of the *in vitro* 3D skin tissue with juvenile human epidermis (see Fig. 1). The protocol, which was pre-validated by ECVAM and accepted by ICH S10, was used with minor modifications that included application of the tested material without a dermatology patch, irradiation of the skin models in DPBS instead of cell culture medium, and introduction of ethanol as an alternative solvent for poorly soluble materials.

Along with TIG-800, two further TiO_2 NPs from commercial sources (AEROXIDE® TiO_2 P25, used as photocatalyst and EUSOLEX T-2000, used as cosmetic UV filter) were tested for comparison. None of the three TiO_2 NP products introduced apparent phototoxicity effects. In line with previous studies (Senzui et al., 2010; Xie et al., 2015; Gaber et al., 2006), the nanoparticles do not penetrate deeply enough through the stratum corneum and lipid layers of the *in vitro* skin model to cause either acute skin irritation (revealed as cytotoxicity in this test) or phototoxicity.

Interestingly, the lowest concentration of TIG-800 (0.00316%) caused a substantial increase in viability in both irradiated and non-irradiated tissue samples (viability of 129.6% in the non-irradiated and 143.4% in the irradiated tissue samples compared to untreated controls). We have not found a clear explanation for this effect, but we hypothesize that the low concentration of NPs on or just under the stratum corneum could have an indirect stimulatory effect on the metabolic activity and homeostasis of the cells. The effect disappeared with increasing concentrations of the NPs on the surface.

We also observed a minor but apparent trend towards increased tissue viability at higher concentrations of TiO_2 NPs, which is probably related to the photo-protective effect, i.e., reflection of UV light by TiO_2. The most significant photo-protection pattern was seen for EUSOLEX T-2000, which is used in sunscreen cosmetics at concentrations up to 25% (w/v). Eusolex T-2000, a rutile-based nano-titanium dioxide contains alumina and simethicone and provides top broad-band protection with high effectivity in the UVA range. Due to the small size of EUSOLEX T-2000 NPs, a transparent effect is achieved on the skin in comparison to TiO_2 microparticles that have around 200 µm and form an unpopular white film on the skin surface upon use in sun-protective cosmetics. To avoid the photo-protective phenomenon, which would bias our experiments, we limited the highest concentration of NPs tested to 3.16% (w/v).

To ensure, that our test predicts the phototoxicity and irritation (expressed as cytotoxicity) of the studied materials correctly, we chose a series of reference chemicals from previously published studies (Jones et al., 2003; Liebsch et al., 1997, 1999). Overall, we obtained results that were highly reproducible and comparable to the data obtained in the studies conducted almost 20 years ago. This confirms both the long-term reproducibility of the tissue model (Rispin et al., 2006) as well as the robustness of the method and its prediction model (Liebsch et al., 1999).

We selected amongst the reference chemicals the challenging chemical anthracene, which was reported to be underpredicted in some previous studies. Liebsch et al. (1997) reported that in two experiments anthracene gave phototoxic results at concentrations of 2-3%, while in two other experiments, anthracene was classified as non-phototoxic up to 10% (false negative). In that study, SSO was used as the solvent, however, we experienced difficulties in solubilizing anthracene in SSO at much lower concentrations than reported by Liebsch et al. (1997). The phototoxicity of anthracene was examined also by Jones et al. (1999), where tissues were irradiated with the same source of UV/VIS light (solar simulator SOL-500), but equipped with an H2 filter, which allows higher penetration of the UVB spectrum. There, anthracene, this time solubilized in ethanol, induced phototoxicity at already very low concentrations of 0.0316%. Jones concluded that the improvement was likely due to the use of the H2 filter, resulting in a higher dose of UVB that was able to activate the anthracene molecule more efficiently.

Anthracene, however, absorbs also in the UVA part of the spectrum; thus, we hypothesized that the false prediction might have been attributable to another factor. Taking into the account the poor solubility of anthracene in water and SSO, we repeated the experiments with anthracene in ethanol using the H1 filter, which cuts off most of the UVB spectrum. Ethanol is well tolerated by the EpiDerm tissue model in the overnight exposure of up to 25 µL and has been used, e.g., at Beiersdorf, to test poorly soluble materials in the H3D PT for many years (Pfannenbecker, personal communication). As an alternative solvent for poorly soluble materials, we also tested a mixture of acetone-olive oil (4:1) used also for *in vitro* skin sensitization assays.

Ethanol as well as acetone-olive oil (AC:OO) solutions of anthracene worked similarly regarding solubility and resulted in the same dose-response in the irradiated and non-irradiated tissue sample. Anthracene was predicted as clearly phototoxic at 0.01% (see Fig. 5). Based on this experience, we recommend for poorly soluble materials to consider ethanol and/or acetone-olive oil (4:1) mixture as additional solvents in a maximum volume of 25 µL. The false-negative prediction in the study of Liebsch et al. (1997) appears to have been caused by the test substance not being bioavailable in the viable layer of the EpiDerm™ model.

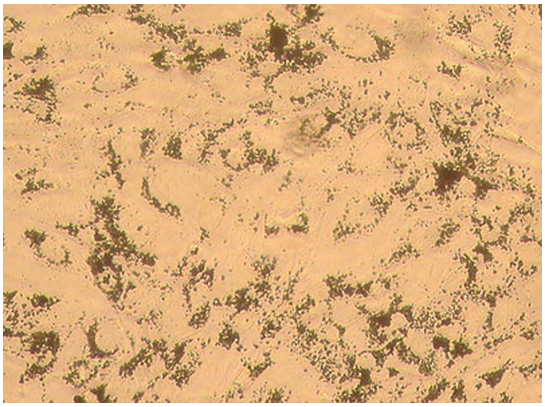

Fig. 6: Cumulation of TiO_2 particles in the cytoplasm of 3T3 mouse fibroblasts
(from Kandárová, 2006)

5 Conclusions

Phototoxicity and cytotoxicity of three structural forms of TiO_2 NPs (AEROXIDE® TiO_2 P25, Eusolex® and TiO_2 TIG-800) were evaluated using the reconstructed human epidermis model EpiDerm™ and the pre-validated phototoxicity test developed by Liebsch et al. (1997).

Despite some published *in vitro* studies pointing at possible photo- and cytotoxicity of the TiO_2 NPs, results obtained in the current study demonstrate that the tested TiO_2 NPs do not induce phototoxic or cytotoxic reactions that would lead to acute phototoxicity or irritation. An explanation of these results could be that the tested nanoparticles do not penetrate deep enough into the viable cells of the reconstructed epidermis to cause cytotoxicity or phototoxicity. This result is supported by the findings of other research groups and by the Scientific Committee on Consumer Safety (SCCS) opinion related to skin toxicity and TiO_2 (SCCS, 2013).

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Conflict of interest

Alžbeta Líšková and Silvia Letašiová are employees of MatTek In Vitro Life Science Laboratories, the manufacturers of the reconstructed human tissue model EpiDerm™ used in the study.

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