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Article

Interactions of TiO_2 Nanoparticles with Ingredients from Modern Lifestyle Products and Their Effects on Human Skin Cells

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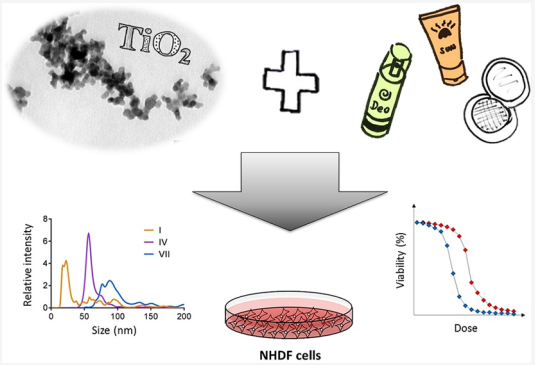
ABSTRACT: The number of consumer products containing nanoparticles (NPs) experienced a rapid increase during the past decades. However, most studies of nanosafety have been conducted using only pure NPs produced in the laboratory, while the interactions with other ingredients in consumer products have rarely been considered so far. In the present study, we investigated such interactions—with a special focus on modern lifestyle products (MLPs) used by adolescents. An extensive survey was undertaken at different high schools all over Austria to identify MLPs that either contain NPs or that could come easily in contact with NPs from other consumer products (such as TiO_2 from sunscreens). Based on the results from a survey among secondary schools students, we focused on ingredients from Henna tattoos (2-hydroxy-1,4-naphtoquinone, HNQ, and *p*-phenylenediamine, PPD), fragrances (butylphenyl methylpropional, known as Lilial), cosmetics and skin-care products (four different parabens). As a cellular model, we decided to use neonatal normal human dermal fibroblasts (nNHDF), since skin contact is the main route of exposure for these compounds. TiO_2 NPs interacted with these compounds as evidenced by alterations in their hydrodynamic diameter observed by nanoparticle tracking analysis. Combinations of TiO_2 NPs with the different MLP components did not show altered cytotoxicity profiles compared to MLP components without TiO_2 NPs. Nevertheless, altered cellular glutathione contents were detected after incubation of the cells with Lilial. This effect was independent of the presence of TiO_2 NPs. Testing mixtures of NPs with other compounds from consumer products is an important approach to achieve a more reliable safety assessment.



1. INTRODUCTION

Titanium dioxide nanoparticles (TiO_2 NPs) are among the most produced types of nanoparticles, with annual production volumes of more than 3000 tons per year.[1,2] Substantial effort has been undertaken to assess the safety of TiO_2 NPs (and NPs in general); however, most of these studies use pristine NPs synthesized in the lab and do not consider mixtures of NPs with other compounds present in consumer products. Since TiO_2 NPs are frequently present in sunscreens,[3] we focused on this particle type, as it is likely to come in contact with chemicals from modern lifestyle products (MLPs) and rise to coexposure *via* the skin.

In general, the outer layer of the human skin is tough and penetration of inorganic NPs through it is very limited.[4−6] While most studies show that TiO_2 NPs do not penetrate the intact or even damaged skin,[7,8] a paper by Wu and co-workers showed penetration of TiO_2 NPs through the nondamaged skin of hairless mice.[9] In the latter study, increased amounts of titanium were observed in different organs such as heart, liver, spleen, and brain. Furthermore, the authors detected significant alterations in malondialdehyde and superoxide dismutase levels in the liver of TiO_2 NP-exposed mice.[9] In a recent study on humans, Pelclova et al. detected TiO_2 NPs in plasma and urine samples 6−48 h after sunscreen use, demonstrating that detectable amounts of the particles can pass the protective layers of the skin.[10]

In addition to *in vivo* studies, several *in vitro* approaches have been undertaken to study the effects of TiO_2 NPs on skin cells. Wright and colleagues studied the effects of differently sized TiO_2 NPs on a human keratinocyte cell line (HaCaT) and concluded that all forms of TiO_2 NPs tested lead to a dose-dependent increase in superoxide production, caspase 8 and 9 activity, and apoptosis.[11] In another study by Crosera et al., the authors showed that TiO_2 NPs induce cytotoxic effects on HaCaT cells with EC_{50} concentrations in the range 10^{-4}−10^{-5}

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mol/L.[7] An earlier study by Pan and co-workers showed that TiO_2 NPs are taken up by primary human dermal fibroblasts and lead to a decrease in cell area, proliferation, mobility, and the ability to contract collagen.[12] It has to be noted that in all of these studies, pure lab-synthesized TiO_2 NPs were used.

In reality, the dermal encounter of TiO_2 NPs occurs for consumers in combination with other external stressors, such as UV light or chemicals. While combinations of TiO_2 NPs and UV light have been examined previously,[13,14] there is a lack of data on the combinatorial effects of TiO_2 NPs with other ingredients from consumer products, e.g., modern lifestyle products (MLPs) such as henna tattoos or certain fragrances or skin-care products. Coexposure of the skin to such MLP ingredients in combination with TiO_2 NPs (for example, from sunscreens) is highly likely and needs to be investigated.

Temporary black henna tattoos became fashionable during the past 15 years among adolescents and are especially applied in holiday areas such as southern European countries by street tattoo artists or at festivals and fairs.[15,16] While the natural orange henna pigments extracted from the plant *Lawsonia inermis* is normally considered as not dangerous, temporary black henna tattoos often contain other ingredients such as the organic compound *para*-phenylenediamine (PPD), which is known to cause hypersensitivity reactions and lead to severe skin damage.[17] In addition, it could be shown *in vitro* that PPD induces dose-dependent cytotoxicity, oxidative stress and altered mRNA expression levels in normal human hair dermal papilla cells.[18] Whether a coexposure of PPD with nanoparticles can lead to enhancement of these effects has not been studied so far.

In addition to tattoos or body-paintings, the skin encounters several other chemicals, e.g., from cosmetics or fragrances. One example is the compound 3-(4-*tert*-butylphenyl)-2-methylpropanal, better known as Lilial, which is discussed to cause contact dermatitis[19,20] and induces dose-dependent toxicity, a decrease of cellular ATP content, and an increase of reactive oxygen species (ROS) production in HaCaT cells.[21] Other critical compounds of cosmetic products are the so-called parabens (esters of parahydroxybenzoic acid), which are used as preservatives in different consumer products, especially in cosmetics.[22] Parabens can penetrate the skin and are discussed to have estrogenic activity and even carcinogenic potential.[22,23] In addition, parabens have been shown *in vitro* to induce cytotoxicity and/or genotoxicity.[24,25]

To our knowledge, no study is available so far that investigates the combinatorial effects of TiO_2 NPs with one of these aforementioned substances on human skin cells. However, since the simultaneous encounter of the human skin to both, TiO_2 NPs, and other compounds from cosmetics, fragrances, or henna tattoos is highly likely, we decided to investigate how these combinations could interact regarding both the characteristics of the NPs and the biological response of human skin cells. We thereby focused on pairwise testing of the individual substances with/without TiO_2 NPs and excluded combinations of multiple substances with the NPs (except for the henna dyes, where we also tested black henna, which contained both, HNQ and PPD). This strategy was chosen since we are particularly interested in the influence of the TiO_2 NPs on potential harmful effects of the substances on human skin, rather than on combinations of several substances. As end points, we decided to use the classical markers for cell stress and cytotoxicity (metabolic activity, lysosomal integrity, apoptosis, oxidative stress) in order to reveal potential harmful effects of the TiO_2/MLP combinations on a basic mechanistic level. A more detailed analysis on potential allergenic or inflammatory effects (due to the substances itself or to contaminations with endotoxins or allergens) was not part of our study but will be interesting for future work in the field.

2. MATERIALS AND METHODS

Materials. Fetal bovine serum (FBS) was purchased from Biochrom (Berlin, Germany), CellTiter-Blue reagent from Promega (Madison, WI, United States), and carboxy-2′,7′-dichlorodihydrofluorescein diacetate and fluorescein-conjugated Annexin V from Thermo Fisher Scientific (Waltham, MA, United States). All other chemicals were obtained from Sigma-Aldrich (Vienna, Austria) at high purity. This includes the tested ingredients from the modern lifestyle products (MLP ingredients), which were purchased as pure chemicals (>96% purity, details see Table 1).

Table 1. Ingredients from Modern Lifestyle Products (MLPs) Used for Investigation of Their Effects on Human Skin Cells in Combination with TiO_2 NPs[a]

compound	purity (%)	origin	concentration (mM)
2-hydroxy-1,4-naphtoquinone (HNQ)	≥97	henna tattoos	2
para-phenylenediamine (PPD)	≥98	black Henna tattoos, hair tinting lotions	0.5
HNQ + PPD	≥97 ≥ 98	black Henna tattoos	0.25 + 0.25
3-(4-*tert*-butylphenyl)-2-methylpropanal (Lilial)	≥96	fragrance used in different cosmetics and skin-care products	0.125
methyl parabene (MP)	≥99	preservative in skin-care products	2
ethyl parabene (EP)	≥99	preservative in skin-care products	1
propyl parabene (PP)	≥99	preservative in skin-care products	0.5
butyl parabene (BP)	≥99	preservative in skin-care products	0.25

[a]The substances were selected from an extensive survey of the use of different MLPs by 252 adolescents in high schools in Austria. Compounds were ordered from Sigma-Aldrich (Vienna, Austria) with the indicated purities. The concentrations given in the last column reflect the concentrations used for the experiments in the present study and are determined as sub-toxic concentrations from dose–response analysis (see Figure 4 and Table 3).

Nanoparticles. Titanium dioxide nanoparticles (TiO_2 NPs) were prepared using a metal organic chemical vapor synthesis approach, as described previously.[26−28] To disperse TiO_2 NPs in water, 2 mg of particles were suspended in 2 mL of H_2O in an Eppendorf cup, vortexed, and subsequently sonicated for 30 min using the UP200St equipped with an S26d2 sonotrode (Hielscher, Teltow, Germany).

Modern Lifestyle Products. Different modern lifestyle products (MLPs) were chosen to study their interactions and combinatorial effects with TiO_2 NPs. The decision for the products and their ingredients tested relies on an extensive survey that was performed on different high schools all over Austria. In detail, data collection was carried out in a two-step process containing (i) a theme processing at school and (ii) an individual online questionnaire. The online survey was divided into the categories, (a) cosmetics, (b) food products, (c) fitness and hobby, (d) party, and (e) others. Test persons should name products of daily use for each category, their common purpose, as well as unusual handling of MLPs. Data evaluation was conducted based on the qualitative content analysis of Mayring, a systematic method used to analyze linguistic material and texts.[29] In accordance

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with Mayring, a process model was developed to split the data analysis into single steps and to generate a transparent and traceable structure.

Characterization of TiO_2 NPs and Their Interactions with MLP Ingredients. TiO_2 NPs and their interactions with MLP ingredients were investigated using nanoparticle tracking analysis (NTA).[30] This method allows for the determination of hydrodynamic particle sizes in dispersion with the advantage of a higher resolution of multiple peaks in polydisperse NP samples compared to standard DLS methods.[30] In addition, NTA is able to estimate particle numbers in samples. In order to determine NP−MLP-ingredient interactions, a mixture of 0.1 mg/mL TiO_2 NPs with 0.1 mg/mL of the respective MLP ingredient was prepared and incubated for 60 min on a rotator to prevent sedimentation. The sample was then diluted with pure H_2O by a factor of 100 and injected into the measurement cell of the NanoSight LM10 (Malvern Instruments, Malvern, United Kingdom). For measurement, five videos of each 30 s duration were recorded and analyzed according to the manufacturer's instructions. Three independent measurements were performed on individually prepared samples.

Cell Culture. Neonatal normal human dermal fibroblasts (nNHDF) cells were purchased from Clonetics (Walkersville, MD, United States) and cultured in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% FBS, 100 units/mL Penicillin, 100 μg/mL Streptomycin, 2 mM L-Glutamine, and 5 mL of Nonessential amino acid solution. Cells were passaged twice per week using standard sterile cell culture techniques. For incubation experiments with TiO_2 NPs and/or MLP ingredients, 38000 viable cells were seeded in 1 mL of culture medium per well of a 24-well plate and grown for 24 h. Cells of passage numbers between 6 and 20 were used.

Experimental Incubation. Cells were taken out of the incubator, and the media were aspirated using a suction pump (Vacuubrand, Wertheim, Germany). Freshly prepared solutions (500 μL) of TiO_2 NPs and/or MLP ingredients in the desired concentrations were added, and the cells were incubated for the desired time points. After incubation, the media were collected and the cells were washed twice with 1 mL of phosphate buffered saline (PBS) before further investigation.

Cytotoxicity Evaluation. The cytotoxicity of MLP ingredients in absence or presence of TiO_2 NPs was analyzed by measuring two different end points (CellTiter-Blue (CTB) assay and Neutral Red Uptake (NRU) assay), as described previously.[31]

Apoptosis Assay. Apoptosis was determined using the Annexin V/Propidiumiodie (PI) staining method as described in Crowley et al.,[32] which was slightly modified. Cells were detached from the plates with 150 μL of trypsin−EDTA solution, washed once with 500 μL of PBS and once with 500 μL of Annexin binding buffer (BB: 10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH = 7.4), and then incubated for 30 min with 150 μL of BB containing 1.5 μL of FITC conjugated Annexin V antibody at room temperature in the dark. After incubation, 1 mL of BB was added and the cells were washed again with 500 μL of BB. Cells were then resuspended in 500 μL of BB containing 2 μg/mL PI, incubated at room temperature, and analyzed by flow cytometry for their fluorescence. Cells incubated with 1 μM staurosporine were used as positive control.

Reactive Oxygen Species Assay. Intracellular reactive oxygen species (ROS) were stained and quantified using the dye 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCFDA). One hour before the end of incubation of the cells with TiO_2 NPs and/or MLP ingredients, 5 μL of a 1 mM solution of DCFDA in DMSO was added to the media and mixed carefully. After the incubation, the cells were washed twice with PBS, detached from the plates by 150 μL of trypsin−EDTA solution, and resuspended in 500 μL of culture medium. Cell suspensions were analyzed by flow cytometry for their fluorescence (FITC channel) using the FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, United States). Hydrogen peroxide (H_2O_2, c = 100 μM) was used as a positive control.

Glutathione Assay. Intracellular total and oxidized glutathione was determined by the earlier published Tietze method,[33] which was adapted to microtiter plates. Briefly, cells were lysed in 200 μL of 1% (w/v) sulfosalicylic acid (SSA), and 10 μL of the lysates were diluted with 90 μL of H_2O and subsequently mixed with 100 μL of a reaction mixture containing 0.3 mM DTNB, 0.4 mM NADPH, 1 mM EDTA, and 1 U/mL glutathione reductase in 0.1 mM sodium phosphate buffer, pH = 7.5. The absorbance at 405 nm was continuously recorded over a time frame of 10 min. Glutathione concentrations were determined by comparing the increase in absorption of samples with the increase in absorption of standards with known concentrations. In order to determine the amount of oxidized glutathione (glutathione disulfide, GSSG), the samples were pretreated with 2-vinylpyridine (2-VP). The sample (cell lysate in SSA, 130 μL) was mixed with 5 μL of 2-VP, and the pH was subsequently adjusted to 6 using 0.2 M Tris-solution. Samples were incubated at room temperature for 60 min, and then 10 μL of the samples were assayed for glutathione, as described above.

Determination of Protein Contents. Cellular protein contents were determined after lysis of the cells in 200 μL of 0.5 M NaOH according to the Lowry method[34] using bovine serum albumin as a standard.

Statistical Analysis. If not stated otherwise, the data represent mean values ± standard deviation (SD) of a minimum of three experiments performed on different passages of cells with individually prepared solution of TiO_2 NPs and/or MLP ingredients. Analysis of significances of differences between two sets of data was performed by the unpaired *t*-test, while groups of data were analyzed using ANOVA with Bonferroni's *post hoc* test (multiple comparisons) or Dunnett's *post hoc* test (multiple comparisons to a control). A *p*-value larger than 0.05 was considered as not significant.

3. RESULTS

Identification of Modern Lifestyle Products (MLPs). Modern lifestyle products (MLPs) used in this study were identified by an extensive survey at seven different high schools all over Austria. In detail, the data evaluation of 252 collected data sets based on the qualitative content analysis of Mayring revealed 390 MLPs in five different categories, cosmetics, fitness and hobby, food products, party, and other. Since most mentioned MLPs referred to the category of cosmetics (305 nominations), this category was further divided into different subcategories (Figure 1). More than 75% of the nominations belonged to hair-care, skin-care, or decorative cosmetics, while other products such as perfumes only represented a minority.

Since the main exposure route for the identified MLPs was the transdermal route *via* the skin (84% of the MLPs, Figure 2), we decided to focus on these products and their ingredients for the current study. Seven different substances that had been

Figure 1. Distribution of the surveyed modern lifestyle products (MLPs) of the category cosmetics. From 390 total MLPs mentioned in the survey, 305 belong to the category of cosmetics and distribute to the presented subcategories.

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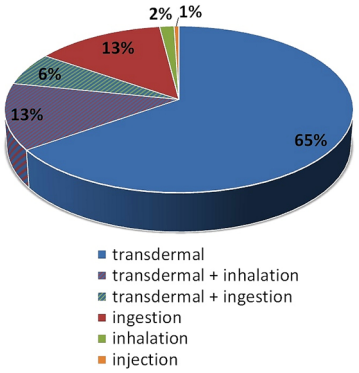

Figure 2. Percentage distribution of exposure routes of surveyed modern lifestyle products (MLPs).

previously shown to be of concern were selected to study their effects alone or in combination with TiO_2 NPs on human skin cells. These substances and their origin are listened in Table 1.

Nanoparticles and Their Interactions with MLP Ingredients. Anatase TiO_2 NPs were synthesized and characterized as previously described.[31,35] The primary particle size was investigated by transmission electron microscopy and was determined as 12 ± 3 nm. Dispersed in water, TiO_2 nanoparticles tend to form small agglomerates with average diameters of 192 ± 3 nm, as measured previously by dynamic light scattering.[31] Detailed analysis of the size distribution of the TiO_2 NPs by nanoparticle tracking analysis (NTA) revealed a rather bell-shaped curve with hydrodynamic diameters of around 50−250 nm for most of the particles (Figure 3). Calculations on the size distribution reveal mean, mode, and D50 values of 130 ± 13, 107 ± 13, and 122 ± 12 nm, respectively (Table 2). Pretreatment of TiO_2 NPs with different MLP ingredients did not lead to any significant changes in their mean, mode, or D50 values; however, there was a significant reduction in detectable particle number from (1268 ± 542) × 10^6 particles in the control condition to (498 ± 380) × 10^6 particles in the sample incubated with HNQ and to (295 ± 325) × 10^6 particles in the sample incubated with

Table 2. Particle Diameters (Mean, Mode and D50 Values) and Particle Numbers of TiO_2 NPs after Incubation with MLP Ingredients[a]

	mean (nm)	mode (nm)	D50 (nm)	# of particles (× 10^6)
control	130 ± 13	107 ± 13	122 ± 12	1268 ± 542
HNQ	154 ± 14	117 ± 18	141 ± 20	498 ± 380*
PPD	138 ± 19	122 ± 14	129 ± 17	1833 ± 93
HNQ + PPD	180 ± 69	176 ± 118	170 ± 88	295 ± 325**
Lilial	136 ± 5	117 ± 11	127 ± 6	552 ± 248
MP	131 ± 2	106 ± 5	122 ± 1	927 ± 146
EP	132 ± 5	105 ± 8	122 ± 2	764 ± 92
PP	126 ± 1	103 ± 5	119 ± 2	612 ± 210
BP	136 ± 18	111 ± 19	122 ± 12	748 ± 137

[a]TiO_2 NPs were incubated with MLP ingredients, and their size distribution was measured using NTA as shown in Figure 3. Statistical mean, mode, and D50 values as well as particle numbers were calculated. The data represent mean values ± SD of three individual performed measurements with individual sets of particles and MLP ingredients. Stars indicate the significance of differences between control conditions (TiO_2 NPs only) and TiO_2 NPs mixed with MLP ingredients; *p < 0.05, **p < 0.01.

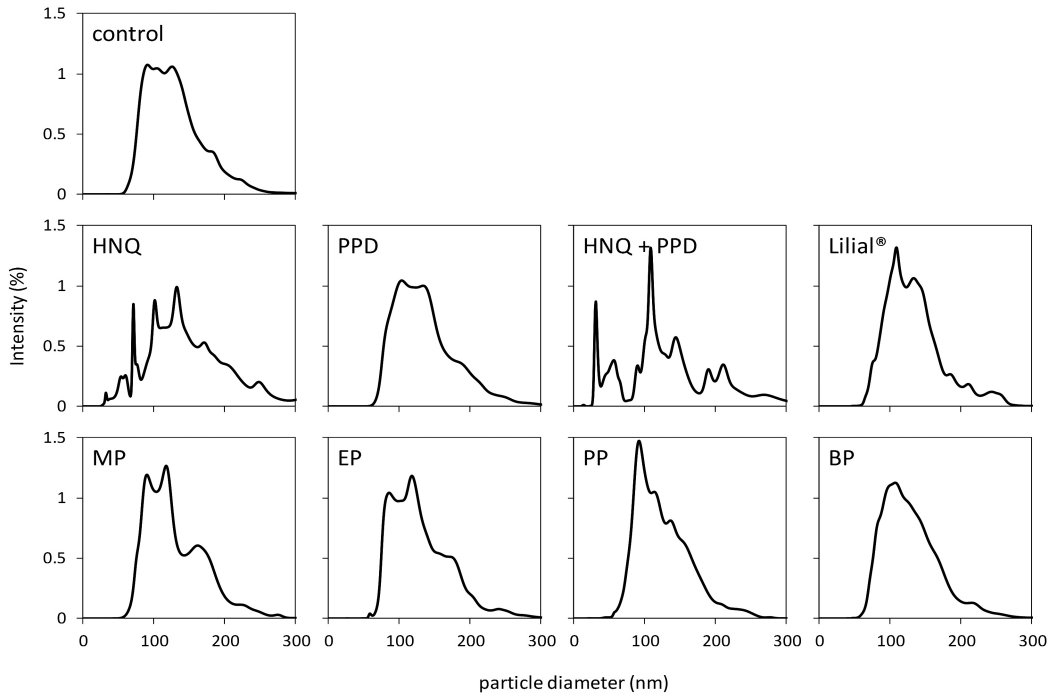

Figure 3. Particle size distribution curves of TiO_2NPs after incubation with MLP ingredients. TiO_2 NPs (0.1 mg/mL) were mixed with 0.1 mg/mL of 2-hydroxy-1,4-naphtoquinone (HNQ), *para*-phenylenediamine (PPD), HNQ + PPD, Lilial, methyl parabene (MP), ethyl parabene (EP), propyl parabene (PP), or butyl parabene (BP) and incubated for 60 min on a rotator. Ten microliters of the mixtures was diluted with 990 μL of pure water, and the particle size distribution was determined using NTA. The size distribution curves represent mean values of three experiments performed with individually prepared samples.

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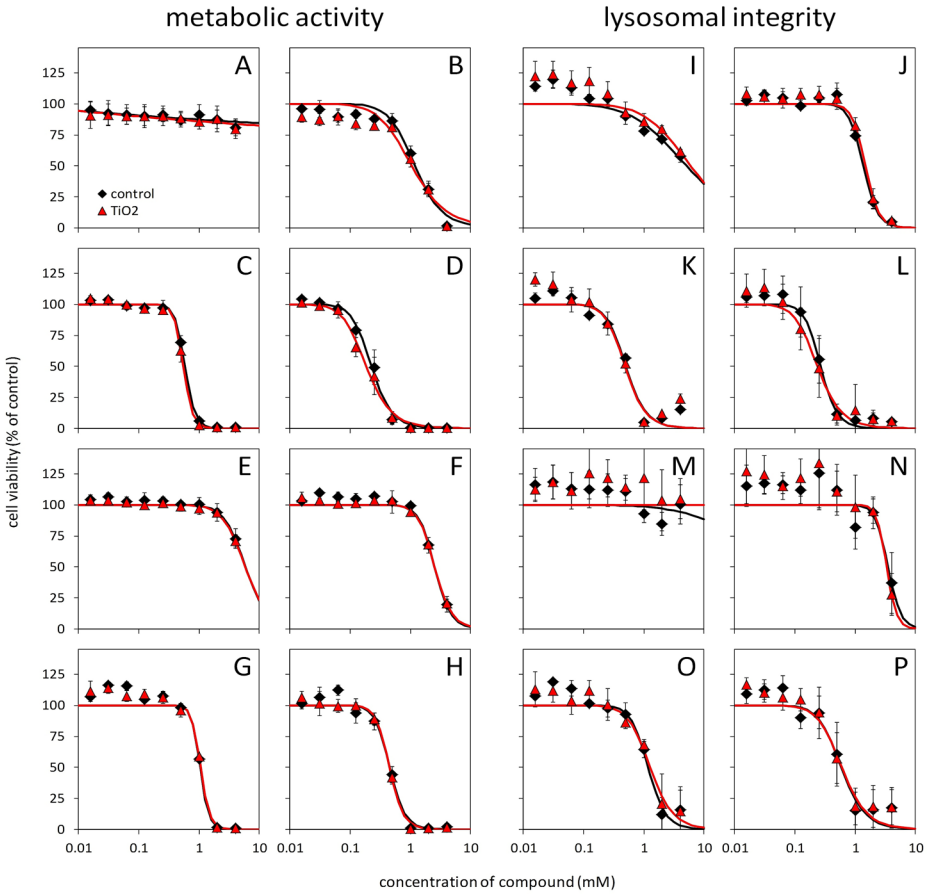

Figure 4. Cytotoxicity of different MLP ingredients in absence or presence of TiO_2 NPs. The cells were incubated for 24 h with various concentrations of 2-hydroxy-1,4-naphtoquinone (HNQ, A, I), *para*-phenylenediamine (PPD, B, J), HNQ + PPD (C, K), Lilial (D, L), methyl parabene (E, M), ethyl parabene (F, N), propyl parabene (G, O), or butyl parabene (H, P) in absence (control) or presence (TiO_2) of 100 μg/mL TiO_2 NPs. After incubation, the cell viability was assessed by determination of the metabolic activity (CTB assay, A–H) and the lysosomal integrity (NRU assay, I–P). Cell viability is always expressed as %viability compared to control cells (treated with cell culture medium only). The data represent mean values ± SD of three different experiments performed individually on independently prepared cultures.

HNQ and PPD (Table 2). These alterations in total detectable particle number are also reflected in the size distribution curves, which show strong differences in their appearance in these two conditions compared to the control or to samples incubated with the other MLP ingredients (Figure 3).

Cytotoxicity of MLP Ingredients. In order to determine the cytotoxicity of the MLP ingredients and to reveal the influence of TiO_2 NPs, a detailed dose–response analysis was performed using nNHDF cells as a model. Incubation of the cells for 24 h with increasing concentrations (0.125–4 mM) of MLP ingredients revealed typical sigmoidal dose–response curves for most of the substances in both, absence or presence of 100 μg/mL TiO_2 NPs (Figure 4). Only for HNQ (Figure 4A) and for MP (Figure 4M), no sigmoidal-shaped dose–response curves were obtained, indicating that for these substances, even the highest concentration tested did not lead to a substantial alteration of the respective readout (metabolic activity, Figure 4A, or lysosomal integrity, Figure 4M). For all substances tested, no influence of the presence of 100 μg/mL TiO_2 NPs was detectable. This is also reflected in the EC50 values, which were calculated from the dose–response curves using a 4-parameter nonlinear regression analysis (Table 3). EC50 concentrations for the different MLP ingredients were between 0.27 ± 0.07 mM (Lilial) and >10 mM (HNQ) and did not significantly alter when TiO_2 NPs were present.

Table 3. EC50 Values Determined on Exposure of nNHDF Cells with Different Compounds in Absence (Control) or Presence (TiO_2) of 100 μg/mL TiO_2 NPs for 24 h

		EC50 (mM)	
compound	end point	control	TiO_2
HNQ	metabolic activity	>10	>10
	lysosomal integrity	5.28 ± 1.09	6.18 ± 0.85
PPD	metabolic activity	1.19 ± 0.17	1.06 ± 0.20
	lysosomal integrity	1.36 ± 0.05	1.48 ± 0.17
HNQ + PPD	metabolic activity	0.58 ± 0.02	0.54 ± 0.04
	lysosomal integrity	0.5 ± 0.03	0.48 ± 0.07
Lilial	metabolic activity	0.23 ± 0.05	0.19 ± 0.05
	lysosomal integrity	0.27 ± 0.07	0.25 ± 0.09
MP	metabolic activity	9.28 ± 7.28	6.31 ± 2.11
	lysosomal integrity	>10	>10
EP	metabolic activity	2.55 ± 0.26	2.55 ± 0.03
	lysosomal integrity	3.54 ± 0.86	3.34 ± 0.6
PP	metabolic activity	1.04 ± 0.02	1.05 ± 0.02
	lysosomal integrity	1.27 ± 0.34	1.44 ± 0.49
BP	metabolic activity	0.45 ± 0.05	0.45 ± 0.03
	lysosomal integrity	0.69 ± 0.42	0.7 ± 0.35

For a more detailed investigation of the effects of MLP ingredients in combination with TiO_2 NPs, subtoxic

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concentrations of the MLP ingredients were chosen. These concentrations are in the range of the EC20 values (i.e., ~80% cell viability) and are depicted in Table 1. In order to study the mechanism of potential loss in cell viability in more detail, a combined apoptosis/necrosis assay was performed using flow cytometry after staining of the cells with the FITC-labeled Annexin V antibody and the dye Propidiumiodide (PI). Differentiation between viable, apoptotic, and necrotic cells was achieved by applying a 4-quadrant gating strategy using a threshold of >95% viable cells in the negative control (NC, Figure 5). As expected from the chosen concentrations of MLP

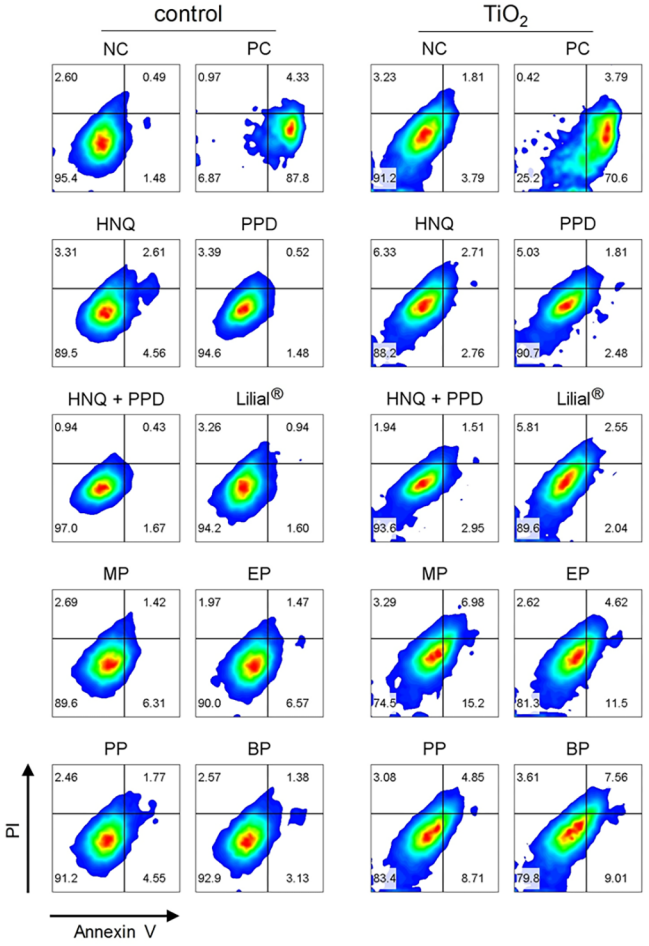

Figure 5. Apoptosis assay of different MLP ingredients in absence or presence of TiO_2 NPs. nNHDF cells were incubated for 24 h with different MLP ingredients (for concentrations see Table 1) in absence (control) or presence (TiO_2) of 100 μg/mL TiO_2 NPs. After incubation, apoptosis was determined using FITC-labeled Annexin V, while necrosis was determined using Propidiumiodide (PI). The data show the results from flow cytometry of a representative experiment reproduced two times with comparable results. NC, negative control; PC, positive control (1 μM staurosporine).

ingredients, the amount of viable cells was >80% for most of the substances tested, while only two of the parabens (MP, BP) lead to slightly reduced numbers of viable cells (74.5% and 79.8%, respectively). Quantification of the fluorescence data on both channels (FITC and PI) revealed that neither the differences between the NC and the MLP-exposed cells nor the differences between control and TiO_2 NP-exposed cells reach the level of significance (Figure 6). Only in the case of PPD together with the TiO_2 NPs was a significant difference in

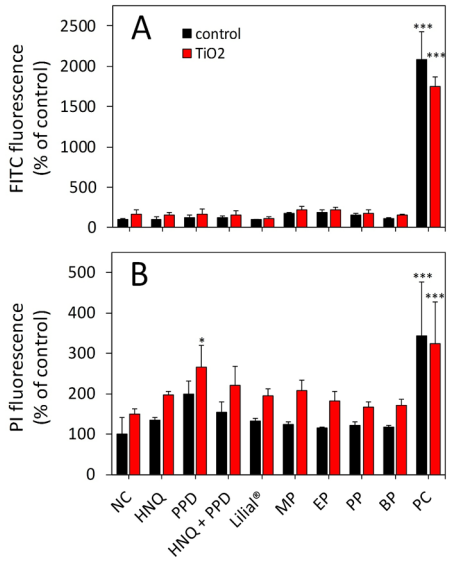

Figure 6. Quantification of apoptosis and necrosis in nNHDF cells after incubation with MLP ingredients in absence or presence of TiO_2 NPs. The cells were incubated for 24 h with different MLP ingredients (for concentrations see Table 1) in absence (control) or presence (TiO_2) of 100 μg/mL TiO_2 NPs and assayed for apoptosis using FITC-labeled Annexin V (A) and necrosis using Propidiumiodide (B). The bar charts show median fluorescence intensities ± SD from flow cytometry data (see Figure 5) given as percentage of negative control (NC) of three independently performed experiments. Stars indicate the significance of differences of exposed conditions compared to the negative control (NC); $*p < 0.05$, $***p < 0.001$. PC: positive control (1 μM staurosporine).

PI fluorescence compared to the control observed. In addition, there is a clear trend that TiO_2 NP-exposed cells always show a higher PI fluorescence than control cells (Figure 6B).

Reactive Oxygen Species Assay. To determine the potential of the MLP ingredients and/or TiO_2 NPs to induce oxidative damage, a staining for reactive oxygen species (ROS) was performed and analyzed by flow cytometry using a double detection method (side-scatter vs. fluorescent signal, Figure 7) and a fluorescent signal quantification method (Figure 8). There was no severe elevation in the DCFDA signal in any of the exposed conditions, with or without TiO_2 NPs, indicating no substantial amount of ROS formed in the cells. Only the positive control (PC: 100 μM H_2O_2) showed a slight but significant increase in the DCFDA signal (Figure 8), demonstrating that the ROS assay was functional in nNHDF cells—even though with a low sensitivity. Nevertheless, a significant difference between positive and negative control was demonstrated, so DCFDA assay was suitable for ROS determination in our setting. The discrepancies between the amount of "ROS positive" events (gating strategy, Figure 7) and median fluorescence intensities (Figure 8) are thus a result of applying two different quantification methods. In addition to the fluorescence signal of the dye, also the side scatter signal (SSC) was recorded as an indicator for cell granularity and, hence, particle accumulation.[36] The data show that there was a significant increase in SSC for all the conditions containing TiO_2 NPs compared to the controls, indicating TiO_2 NP accumulation in cells independent of the MLP ingredients.

Cellular Glutathione Content. As an additional marker for cellular (oxidative) stress, the total cellular glutathione (GSH) content as well as the amount of oxidized glutathione

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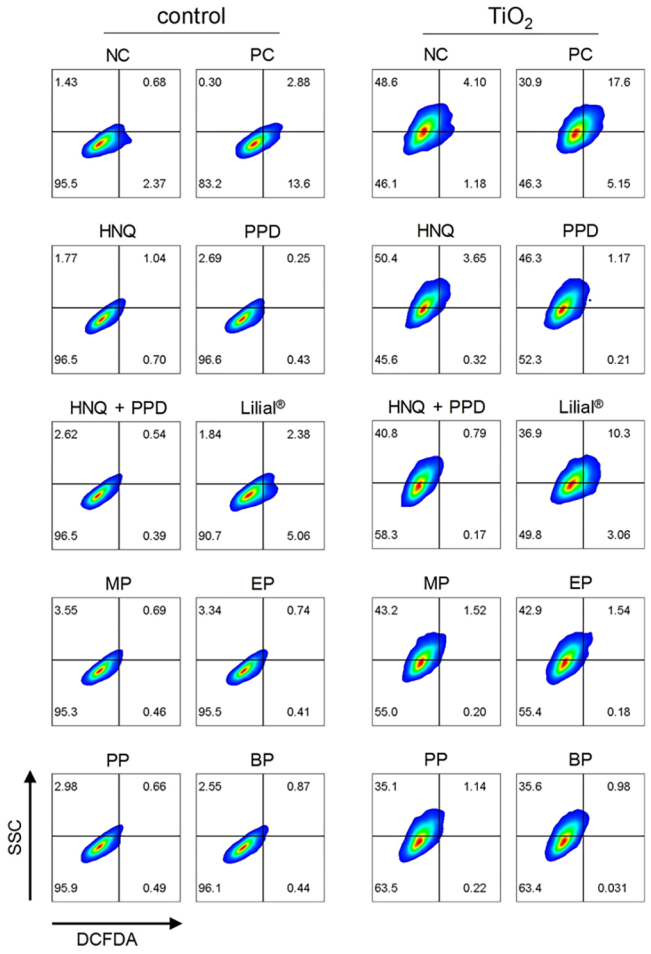

Figure 7. Reactive oxygen species (ROS) assay of different MLP ingredients in absence or presence of TiO_2 NPs. nNHDF cells were incubated for 4 h with different MLP ingredients (for concentrations see Table 1) in absence (control) or presence (TiO_2) of 100 μg/mL TiO_2 NPs. After incubation, ROS were detected using carboxy-dichlorodihydrofluorescein diacetate (DCFDA). In addition, the side scatter signal (SSC) was monitored as a measure of TiO_2 particle accumulation. The data show the results from flow cytometry of a representative experiment reproduced two times with comparable results. NC, negative control; PC, positive control (100 μM H_2O_2).

(glutathione disulfide, GSSG) was monitored (Figure 9). After 4 h of incubation of nNHDF cells with cell culture medium only, the total cellular glutathione content was 36.2 ± 2.8 or 34.6 ± 2.0 nmol/mg protein in absence or presence of TiO_2 NPs, respectively. These values remained rather unaffected when the cells were incubated with most of the MLP ingredients but were lowered significantly when the cells were incubated with 100 μM H_2O_2 as the positive control. However, the treatment with 0.5 mM PP in the absence of TiO_2 NPs and the treatment with 0.125 mM Lilial—in absence or presence of TiO_2 NPs—resulted in a significantly lower total cellular glutathione content (Figure 9). For Lilial, the resulting cellular glutathione contents were 23.4 ± 2.0 or 22.7 ± 2.7 nmol/mg protein in absence or presence of TiO_2 NPs, respectively. The cellular contents of GSSG remained very low (<1 nmol/mg, which is close to the detection limit of the assay) and was unaffected in all the experimental conditions.

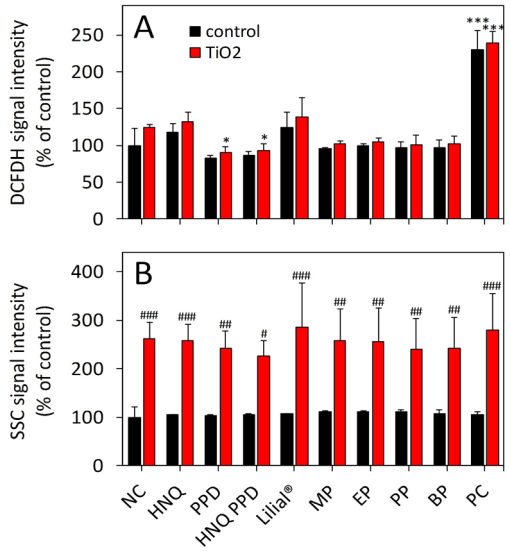

Figure 8. Quantification of reactive oxygen species (ROS) and particle accumulation in nNHDF cells after incubation with MLP ingredients in absence or presence of TiO_2 NPs. The cells were incubated for 24 h with different MLP ingredients (for concentrations see Table 1) in absence (control) or presence (TiO_2) of 100 μg/mL TiO_2 NPs and assayed for ROS using DCFDA (A) and TiO_2 NP accumulation using the SSC signal intensity (B). Bar charts show median fluorescence intensities ± SD from flow cytometry data (see Figure 7) given as percentage of negative control (NC) of three independently performed experiments. Stars indicate the significance of differences of exposed conditions compared to the negative control (NC); $^{*}p < 0.05$, $^{***}p < 0.001$. Hashtags indicate the significance of differences between control and TiO_2 NP-exposed conditions; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$. PC: positive control (100 μM H_2O_2).

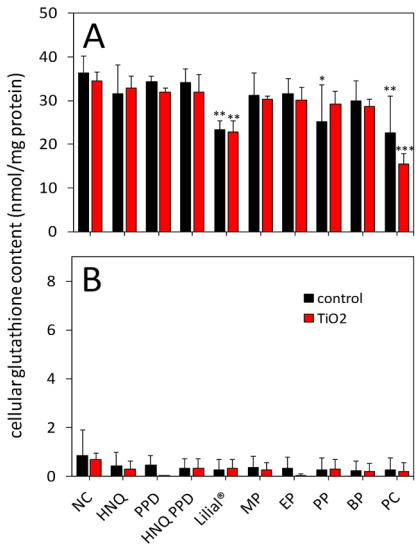

Figure 9. Glutathione quantification after incubation of nNHDF cells with MLP ingredients in absence or presence of TiO_2 NPs. The cells were incubated for 4 h with different MLP ingredients (for concentrations see Table 1) in absence (control) or presence (TiO_2) of 100 μg/mL TiO_2 NPs, and total glutathione content (A) as well as oxidized glutathione disulfide content (B) was measured. The data represent mean values ± SD of three independently performed experiments. Stars indicate the significance of differences of exposed conditions compared to the negative control (NC); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. PC: positive control (100 μM H_2O_2).

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4. DISCUSSION

Titanium dioxide nanoparticles (TiO_2 NPs) are used widely in different consumer products, including sunscreens. Although, the general view describes the skin as a tight barrier, rather impermeable to NPs, there is evidence in the recent literature that some NPs enter the *stratum corneum* or even penetrate the skin completely.[9,10,14,37,38]

The here used TiO_2 NPs were produced by a metal organic chemical vapor synthesis approach leading to pure anatase NPs without contamination with any synthesis residues or other compounds such as bacterial endotoxins (data not shown). The primary size of these particles was between 8 and 18 nm (mean: 13 nm), as reported earlier.[35] The fact that these particles show hydrodynamic diameters of 130 ± 13 nm indicates the formation of smaller particle agglomerates as previously published.[31] Agglomeration or aggregation is a frequently observed characteristic of NPs and is also known to occur for such particles in consumer products as shown for TiO_2 or ZnO NPs in sunscreens[39] or Ag NPs in antibacterial sprays.[40] Although it is evident that the behavior of TiO_2 NPs in pure H_2O cannot be directly compared to the behavior in sunscreens (foams, gels, sprays, powders, etc.) the size-data obtained in this study are comparable with size-data of agglomerates found in the earlier studies of Lu and colleagues on TiO_2 NPs in commercial sunscreen sprays[41] or powders.[39]

Incubation of TiO_2 NPs with different ingredients from modern lifestyle products (MLPs) did not show severe alterations in the NP size distribution curves for most of the MLPs, suggesting no direct interaction between the substances and the particles. However, samples containing the Henna dye 2-hydroxy-1,4-naphtoquinone (HNQ) lead to an intensive change in the TiO_2 NP size distribution and detectable particle number, suggesting a direct interaction of this compound with the surface of the NPs. Most likely, the presence of the dye HNQ fosters the generation of larger agglomerates/aggregates of TiO_2 NPs that cannot be detected properly with the NTA method due to rapid gravitational settling, which would explain the reduced detectable particle number. The small fractions of smaller particles visible in the size distribution curves are likely to represent artifacts due to large glaring particles identified from the NTA video images as shown earlier for protein aggregates.[42] It has recently been shown, that extracts from the Henna plant *Lawsonia inermis* can be used for green synthesis of Ag or CeO_2 NPs,[43] but no study is currently available that investigates the direct interactions of HNQ with TiO_2 NPs. At least one study showed that HNQ extracted from Henna plants can be used to synthesize colored TiO_2 NPs that contain the dye and can be used for fabrication of dye-sensitized solar cells.[44] It should be noted furthermore that the agglomeration state of TiO_2 NPs influences their performance as an UV filter.[45]

TiO_2 NPs are frequently used as an UV attenuator in different skin-care products such as lotions, sunscreens, and cosmetics. Thus, the encounter of the skin to such particles and other bystander substances from the products or from MLPs used on the skin is evident, and possible combinatorial effects should be investigated. TiO_2 NPs alone did not result in any cytotoxicity when applied in a concentration of 100 μg/mL for 24 h. This supports earlier findings by Browning et al.[46] but contrasts studies of Chang et al.[47] who reported an EC50 of 125.1 ± 23.6 μg/mL for TiO_2-exposed human skin cells. The different MLP ingredients showed a classical dose-dependent cytotoxicity with EC50 values ranging from ∼0.2 to 10 mM dependent on the respective substance. The highest toxicity was induced by Lilial, followed by butyl parabene and the HNQ/PPD mixture. For PPD alone, we determined an EC50 of around 1 mM, which is 10 times higher than recently published data for PPD toxicity on rat skin cells.[48] Butyl parabene was the most toxic compound from the tested parabens followed by propyl, ethyl, and methyl parabene. This strongly suggests that the length of the side chain of the esters of 4-hydrodybenzoic acid plays a role in its cytotoxic potential. Indeed, similar findings were obtained by Lee and colleagues in a recent study on *Daphnia magna* and *Aliivibrio fischeri*.[49] The EC50 values in this study ranged from 73.4 mg/L (0.48 mM) for methyl parabene to 2.34 mg/L (0.012 mM) for butyl parabene. A likely explanation for this fact is that parabens with longer alkyl chains have a reduced water solubility and, thus, likely a better potential for bioaccumulation.

Interestingly, the observed cytotoxicity profiles for all MLP ingredients did not change when 100 μg/mL TiO_2 NPs were present during the incubation. This demonstrates that there is no combinatorial (or at least no additive) effect of TiO_2 NPs with the different substances leading to an increase of cell death. So far, no study is available in the literature that investigates the combinatorial effects of these compounds with TiO_2 NPs on skin cells. In a recent paper, Roszak et al. investigated the combinatorial effects of Ag NPs with butyl parabene on human breast cell lines and concluded that there is no increased genotoxic effect of the combinatorial exposure compared to the exposure with Ag NPs alone.[25]

A combined apoptosis/necrosis assay was undertaken in order to investigate possible combinatorial effects between MLP ingredients and TiO_2 NPs in more detail. This assay also did not show any additive effects of TiO_2 NPs and MLP ingredients on the viability of nNHDF cells. However, we found that TiO_2 NP-treated cells always show a slightly higher level of PI fluorescence compared to control cells. This could be an indicator for an increased loss of membrane integrity under these conditions compared to the control. However, the data did not reach the level of significance. Furthermore, it should be noted that interferences of the TiO_2 NPs with the different cytotoxicity assays can also lead to the differences in the results.[50]

TiO_2 NPs have been shown to cause oxidative stress and lead to reduced cellular glutathione contents in earlier studies on human epidermal cells (A431) and human keratinocytes (HaCaT).[11,51] In the present study on nNHDF cells, neither TiO_2 NP-dependent induction of reactive oxygen species (ROS) nor a reduction in cellular glutathione content were observed. Also the used MLP ingredients did not lead to elevated ROS in nNHDF cells after incubation. HNQ and PPD even decreased the amount of detectable ROS significantly, probably due to interferences of these dyes with the used ROS indicator. Incubation of nNHDF cells with Lilial led to a significant reduction in cellular glutathione content—independently of the presence of TiO_2 NPs. This finding supports an earlier study by Usta and co-workers who demonstrated that HaCaT cells experience a decrease in viability and cellular ATP content and an increase in ROS production after exposure to Lilial.[21] The amounts of oxidized glutathione disulfide (GSSG) remained unaltered for all conditions tested, including the positive control (100 μM H_2O_2), which contrasts earlier findings on HaCaT cells.[52] A

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possible explanation could be the export of oxidized GSSG from the cells as recently shown for cultured brain astrocytes.[53]

Significant increases in side scatter (SSC) signals in all test conditions containing TiO_2 NP compared to conditions without particles strongly suggest TiO_2 NP accumulation/uptake onto or in the cells, as shown in our earlier report on lung epithelial cells.[31] However, to validate these data on nNHDF cells, additional imaging techniques such as confocal laser scanning microscopy or transmission electron microscopy should be applied in future studies.

5. CONCLUSIONS

In summary, our data present for the first time a detailed analysis of the combinatorial effects of TiO_2 NPs with ingredients from modern lifestyle products (MLPs) such as Henna tattoos, skin-care products, and cosmetics. TiO_2 NPs alone did not cause any loss in cell viability when applied in a concentration of 100 μg/mL up to 24 h. The cytotoxicity of the different MLP ingredients exhibits typical sigmoidal dose–response relationship with EC50 values in the low millimolar range. The presence of TiO_2 NPs neither altered the cytotoxicity profiles of the MLP ingredients nor resulted in alterations of cellular apoptosis, ROS production, or cellular glutathione content. Taken together, it can be concluded that there are not additive effects of TiO_2 NPs and the selected ingredients from cosmetics or tattoos, even though the Henna tattoo ingredient 2-hydroxy-1,4-naphtoquinone (HNQ) was able to strongly influence the agglomeration behavior of the NPs. It should be noted here, that the present study only focuses on single pairwise testing of individual compounds and TiO_2; whereas, the real-life situation is likely to be much more complicated, involving several compounds acting together and (potentially) interacting with NPs. We suggest that similar studies should be part of the safety analysis of consumer products when it can be expected that use of an NP-enabled product leads to coexposure with potentially problematic chemicals.

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Notes

The authors declare no competing financial interest.

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LIST OF ABBREVIATIONS

2-VP	2-vinylpyridine
ANOVA	analysis of variances
ATP	adenosine triphosphate
BB	binding buffer
BP	butyl parabene
CTB	CellTiter-Blue
DCFDA	2′,7′-dichlorodihydrofluorescein diacetate
DMEM	Dulbecco's modified Eagle's Medium
DMSO	dimethyl sulfoxide
DTNB	5,5′-dithiobis(2-nitrobenzoic acid)
EDTA	ethylenediaminetetraacetic acid
EP	ethyl parabene
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GSH	glutathione
GSSG	glutathione disulfide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HNQ	2-hydroxy-1,4-naphtoquinone
MLP	modern lifestyle product
MP	methyl parabene
NADPH	nicotinamide adenine dinucleotide phosphate
NC	negative control
nNHDF	neonatal normal human dermal fibroblasts
NP	nanoparticle
NRU	Neutral Red uptake
NTA	nanoparticle tracking analysis
PBS	phosphate buffered saline
PC	positive control
PI	propidium iodide
PP	propyl parabene
PPD	*para*-phenylenediamine
ROS	reactive oxygen species
SD	standard deviation
SSA	sulfosalicylic acid
SSC	side scatter
UV	ultraviolet

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