Investigating the accumulation and translocation of titanium dioxide nanoparticles with different surface modifications in static and dynamic human placental transfer models

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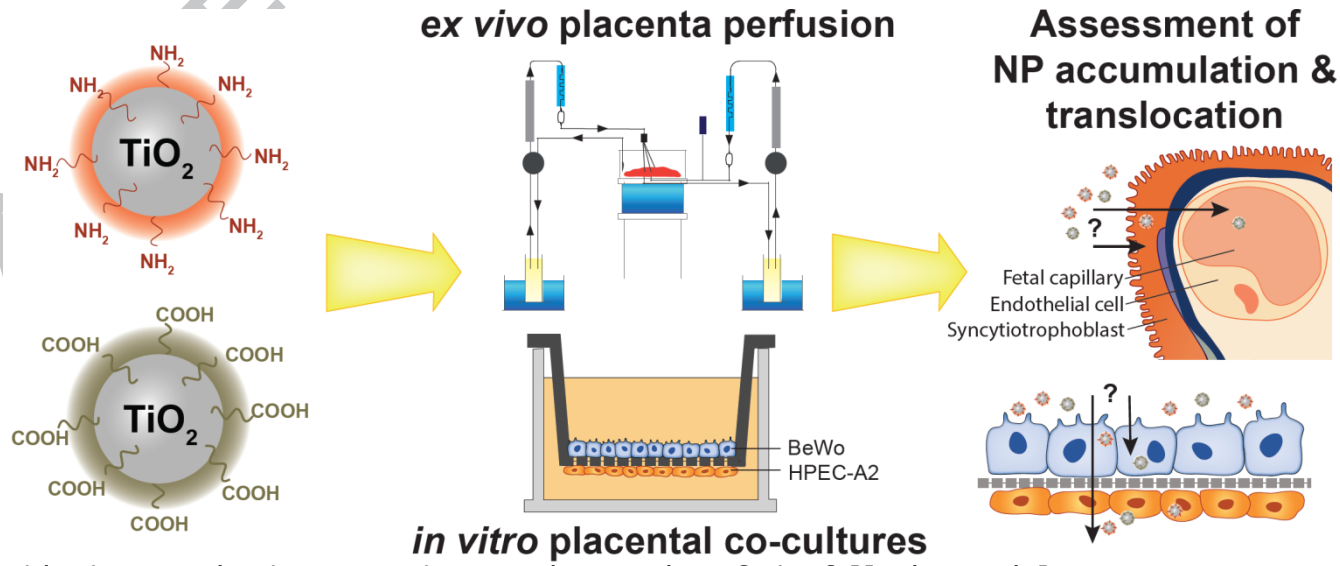
Graphical Abstract



This document is the accepted manuscript version of the following article:
Aengenheister, L., Dugershaw, B. B., Manser, P., Wichser, A., Schoenenberger, R., Wick, P., … Buerki-Thurnherr, T. (2019). Investigating the accumulation and translocation of titanium dioxide nanoparticles with different surface modifications in static and dynamic human placental transfer models. European Journal of Pharmaceutics and Biopharmaceutics, (25 pp.).
https://doi.org/10.1016/j.ejpb.2019.07.018

Abstract

Titanium dioxide nanoparticles (TiO_2 NPs) are widely incorporated in various consumer products such as cosmetics and food. Despite known human exposure, the potential risks of TiO_2 NPs during pregnancy are not fully understood, but several studies in mice elucidated toxic effects on fetal development. It has also been shown that modifying NPs with positive or negative surface charge alters cellular uptake and abolishes fetotoxicity of silicon dioxide (SiO_2) NPs in mice.

Here, we investigated accumulation and translocation of positively charged TiO_2-NH_2 and negatively charged TiO_2-COOH NPs at the placental barrier, to clarify whether surface charge provides a means to control TiO_2 NP distribution at the placental barrier. To ensure outcome relevant for humans, the recently developed *in vitro* human placental co-culture model and the gold standard amongst placental translocation models – the *ex vivo* perfusion of human term placental tissue - were employed during this study. Sector field-ICP-MS analysis of maternal and fetal supernatants as well as placental cells/tissues revealed a substantial accumulation of both TiO_2 NP types while no considerable placental translocation was apparent in both models. Characterization of agglomeration behavior demonstrated a strong and fast agglomeration of TiO_2-NH_2 and TiO_2-COOH NPs in the different culture media.

Overall, our results indicate that surface charge is not a key factor to steer placental uptake and transfer of TiO_2. Moreover, the negligible placental transfer but high accumulation of TiO_2 NPs in placental tissue suggests that potential effects on fetal health may occur indirectly, which calls for further studies elucidating the impact of TiO_2 NPs on placental tissue functionality and signaling.

Keywords

titanium dioxide nanoparticles, *in vitro* human placental co-culture model, *ex vivo* human placenta perfusion, placental accumulation, placental translocation

Introduction

One of the most widely manufactured materials on a global scale is titanium dioxide (TiO_2). In its bulk form, it has been considered a safe and inert material for decades [1] and approved by the European Union as a food additive (E171), which has led to its increasing usage in a broad range of consumer products [2]. However, materials considered to be inert may act differently when introduced to a biological system as nanomaterial [3–5]. Compared to their corresponding bulk materials, nanoparticles (NPs) have at least one dimension ≤100 nm resulting in significantly increased surface-to-volume ratio. Further unique physicochemical, mechanical or optical characteristics include high chemical reactivity, lower melting temperature, electrical conductivity, magnetic permeability and greater solar radiation absorption, among others [6]. The high prevalence of TiO_2 NPs and their unique properties may pose potential risks to human health [7–9]. For instance, based on preclinical inhalation studies, the International Agency for Research on Cancer (IARC) declared TiO_2 NPs as "possibly carcinogenic when inhaled" and highlighted the need to better understand its potential adverse effects by different routes of exposure in humans [10].

Since the thalidomide scandal in the early 60's, it is obvious that pregnant women and their unborn children are among the most vulnerable populations at risk when exposed to xenobiotic substances. Phases of rapid growth and development make fetuses more susceptible to toxic effects than adults [11]. Moreover, profound physiological changes are occurring in pregnancy including increased maternal blood volume, cardiac output, and blood flow to the kidneys and uteroplacental unit [12], which are likely to alter the biodistribution of NPs and increase potential risks for both mother and the developing fetus.

First evidence for potential developmental toxicity of NPs came from epidemiological studies highlighting increased risks for pregnancy complications (e.g. low birth weight or preterm birth) upon maternal inhalation of particulate matter (PM) present in polluted air [13–17]. However, the underlying toxicity mechanisms have not yet been identified. It has been demonstrated that some NPs, including nanosized TiO_2, are capable to cross the placental barrier [18] and reach fetal organs like liver and brain in mice [19]. Placental translocation of TiO_2 NPs was linked to an impairment of normal growth and development of murine fetuses [20] or neural tissue damage [21,22]. Fetotoxicity of TiO_2 NPs has also been reported in the absence of placental transfer, possibly due to

NP interference with placental function or the release of maternal and/or placental mediators caused by oxidative stress and inflammatory reactions [18,23]. For instance, Notter et al. observed neurobehavioral deficits in the offspring of mice while only detecting TiO_2 accumulation in the maternal liver and spleen but not in the placenta, fetal liver or brain [24]. TiO_2 NPs have also been shown to accumulate in the placental tissue and cause tissue damage leading to placental dysregulation and dysfunction in mice [25].

Collectively, the previous studies indicate that at least some of the developmental toxicity induced by TiO_2 NPs may be due to placental accumulation or translocation of particles. Changing the properties or surface modification may provide a means to control cellular uptake and translocation of NPs at biological barriers. For instance, particle size has been identified as a decisive factor in transplacental translocation with higher transfer for smaller particles [18]. Surface charge could be another promising candidate since NP modification with amine and carboxyl groups can affect cellular uptake and has been shown to abolish the fetotoxic effects of silicon dioxide (SiO_2) NPs in pregnant mice by a yet unknown mechanism [19]. However, the role of surface charge on NP transport at the placental barrier has only been assessed in a limited number of studies with conflicting findings [18]. For example, in mice, unmodified as well as amine and carboxyl modified SiO_2 NPs were found in fetal tissue by transmission electron microscopy (TEM) imaging, but no quantitative data was provided on transport rates [19]. Another *in vitro* study demonstrated that the placental transport of polystyrene NPs (PS NPs) is influenced by their surface charge [26]. However, two different types of PS NPs with similar negative surface charge but from different manufacturers displayed completely different translocation behavior. These NP types were either more or less translocated across the placental barrier compared to the positively charged NP type [26]. These examples demonstrate the difficulty of comparing results between and within studies due to variations in e.g. NP characteristics and analytical detection methods. In addition, it highlights that more work needs to be done to identify the influence of NP surface modification on placental transport [18]. Studies on placental transfer of NPs as well as potential adverse effectson the offspring have mainly been performed in rodents. These have led to first insights on potential fetotoxicity of NPs in a living organism. Translation to humans, however, remains challenging since the placenta is the most species-specific organ [27,28].

The aim of this research study was to explore placental uptake, accumulation and translocation of positively charged TiO_2-NH_2 and negatively charged TiO_2-COOH NPs in human-relevant placenta models to avoid species-specific differences. The *ex vivo* placenta perfusion system is the current gold standard for transport studies (up to 6h) across the term placenta [29], but we also employed our recently developed *in vitro* placenta co-culture transfer model [30] for prolonged uptake and transfer studies (up to 24h).

Material & methods

NP preparation and characterization

TiO_2 NPs used in this study were custom-synthesized and supplied by PlasmaChem GmbH (Berlin, Germany) within the BMBF-funded project NanoUmwelt (03X0150) as model TiO_2 NPs to study charge-dependent distribution at the placental barrier. In brief, the core particles with a target size of 4-8 nm were prepared via a sol-gel process where titanium tetrachloride was hydrolyzed, followed by the condensation of all reaction products and their stabilization with HNO_3. The primary particle size of the unmodified TiO_2 NPs was 4-8 nm. TiO_2-NH_2 and TiO_2-COOH NPs were manufactured via the addition of either 3-aminopropyltrimethoxysilane (APTMS) or citric acid, respectively.

TiO_2 NP suspensions were prepared in the same way for all experiments. First, the NP stock suspensions were sonicated for 15 min.For NP characterization, the TiO_2 NP suspensions (0.1 mg/mL) were prepared by gradual dilution in ultrapure water, supplemented endothelial cell medium (EM) orperfusion medium (PM; please see detailed description of the different media below). Zeta potential measurements were performed in disposable folded capillary cells (DTS1070, Malvern, Worcestershire, UK), using a Zetasizer Nano ZS (Malvern, Worcestershire, UK) with 90 s equilibration time and three consecutive measurements (with at least 10 runs and maximum 100 runs). Hydrodynamic diameter measurements were performed in ultrapure water, EM and PM immediately after preparation of the suspensions and after 6 h and 24 h incubation at 37 °C. Importantly, samples were not mixed before each measurement to mimic experimental conditions and study NP agglomeration and sedimentation behavior in the suspensions over time. Samples were measured in disposable PMMA cuvettes (VWR, Darmstadt, Germany) using the Zetasizer system (three consecutive measurements with 12 runs were performed). Hydrodynamic diameters are presented as intensity-based overall average size (Z-average).

In vitro cell barrier formation

Cell culture conditions and placental monolayer and co-culture formation were done as previously described [30]. In brief, Transwell® inserts (polycarbonate, pore size 3.0 µm, growth area 1.12 cm^2, apical volume 0.5 mL, basolateral volume 1.5 mL; Corning®, Sigma-Aldrich, Buchs, Switzerland) were pre-coated with human placental collagen IV

(Sigma-Aldrich, Buchs, Switzerland) for 1 h at 37 °C/ 5% CO_2. Monolayers of BeWo b30 (human placental choriocarcinoma cell line) or HPEC-A2 cells (SV40-transformed microvascular human placental venous endothelial cells) were obtained by seeding 1.5 x 10^5 or 1.0 x 10^5 cells per membrane on the apical or basolateral side of the membrane, respectively. Placental co-culture barriers were prepared by initially seeding HPEC-A2 cells and allowing them to settle on the basolateral side of the membrane for 2 h, followed by cultivating BeWo cells on the apical side. For translocation studies, monolayers and co-cultures were incubated at 37 °C/ 5% CO_2 for 3 d under static conditions (e.g., without shaking or stirring) in supplemented endothelial cell growth medium MV (complemented with SupplementMix according to the manufacturer`s guide,PromoCell, Heidelberg, Germany and 1% penicillin/streptomycin, Gibco, Luzern, Switzerland), which is referred to as "EM" throughout the manuscript. EM was changed after 48 h and before the translocation experiment.

In vitro translocation study

To assess placental translocation across monolayers and co-cultures as well as cellular uptake of TiO_2-NH_2 and TiO_2-COOH NPs *in vitro,* 1 µg/mL of the NPs was given to the apical chamber of the inserts (0.5 mL; EM), whereas fresh EM was added to the basolateral side (1.5 mL; EM). Incubation of the cultures was done at 37 °C/ 5% CO_2 under static conditions. Samples were taken from the basolateral chamber after 6 h (1.5 mL) as well as from both compartments after 24 h (0.5 mL and 1.5 mL), and the volume was replenished with fresh pre-warmed EM. To determine the influence of TiO_2 NPs on cell barrier integrity, the transepithelial electrical resistance (TEER) was measured before and after NP treatment in EM (see supplement figure S3). Afterwards, the membranes were cut out of the insert and all samples (apical, basolateral, membrane) were stored at 4 °C for further analysis.

Cell viability assay

The effect of TiO_2-NH_2 and TiO_2-COOH NPs on the viability of HPEC and BeWo cells cultivated in EM was evaluated using the MTS assay (CellTiter96® AQueous One Solution Cell Proliferation Assay, Promega, Dübendorf, Switzerland). 24 h before the addition of TiO_2 NPs, cells were seeded in 96 well plates (1 x 10^4 cells/ well) and

incubated at 37°C/ 5% CO_2. After 6, 24 and 48 h of NP exposure (0 – 25 µg/mL), the MTS assay was conducted according to the manual. Untreated cells and cells exposed to 1 mM $CdSO_4$ were used as negative and positive control, respectively. Optical density values (490 nm, Mithras2 LB 943, Berthold Technologies GmbH, Zug, Switzerland) were blank-corrected and normalized to the negative control. Interference of the TiO_2 NPs with the test system was excluded in advance (data not shown; cell-free controls as described in [31]).

Ex vivo human placental perfusion

The project was ethically approved and performed according to the principles of the Declaration of Helsinki. Written consent was given by the expecting women before caesarean section at the Kantonsspital and Hirslanden Klinik Stephanshorn in St. Gallen, Switzerland. Placentas from uncomplicated pregnancies were perfused in a closed system as previously described [29]. Briefly, the perfusion medium (PM) was prepared by diluting M199 tissue culture media with Earl`s buffer (1:2) and supplementing it with bovine serum albumin (BSA; 10 g/L), dextran 40 (10 g/L), sodium bicarbonate (2.2 g/L), glucose (1 g/L), amoxicillin (250 mg/L) and sodium heparin (2500 IU/L) (all supplements were purchased from Sigma-Aldrich, Buchs, Switzerland). Experiments were considered valid when the pre-perfusion did not show signs of leakage, the transport of fluid from the fetal to maternal side during the translocation experiments was lower than 4 mL/h, and the pH remained within 7.2 and 7.4. Maternal and fetal samples were taken 0, 0.25, 0.5, 1, 2, 3, 4, 5 and 6 h after adding TiO_2-NH_2 and TiO_2-COOH NPs (10 µg/mL), directly followed by pH measurement with a blood gas analyzer (Epocal Inc., Ottawa, Canada). Placental tissue specimens were taken before and after each perfusion experiment. The amount of freely available TiO_2 NPs during the *ex vivo* perfusion experiments was estimated by closed perfusion of the maternal side (fig. S5; cannulas were put in the maternal cylinder; no placental tissue; similar to [32]). Except for the use of 50 mL PM, the same experimental conditions were used as described before. All samples were stored at -20 °C until further processing.

Sector field-inductively coupled plasma-mass spectrometry (SF-ICP-MS)

The Ti content was determined using SF-ICP-MS (Element 2, Thermo Finnigan, Bremen, Germany). For sample preparation, 1 g placental tissue was homogenized in 3 mL PM (~250 µL equals 83.33 mg tissue) using a TissueRuptor (QIAGEN, Hilden, Germany). All samples (250 µL of *in vitro* supernatants and membranes as well as 250 µL of tissue homogenate, and maternal and fetal flow through from *ex vivo* experiments) were digested in 2 mL concentrated nitric acid (HNO_3, NORMATOM, VWR Chemicals, Vienna, Austria) using a microwave (turboWAVE Inert, MLS GmbH, Leutkirch, Germany). Further dilution of the samples was done with ultrapure water. To avoid potential contamination, all vials which were used for sample preparation were pre-cleaned with 20% HNO_3 for 4 h at room temperature and rinsed twice with ultrapure water. Furthermore, the Ti content was determined using medium resolution via SF-ICP-MS within 24 h after sample preparation. Indium was added as internal standard during all measurements. Quantification of the isotope ^{49}Ti was done with external calibration using matrix-adapted standards ranging from 0-5 µg/L. The detection limits (LOD = $mean_{blank}$ + 3*SD_{blank}) of ^{49}Ti were 0.57 µg/L, 0.44 µg/L, 0.23-0.30 µg/L and 4.99 µg/L in *in vitro* supernatants, membranes, *ex vivo* flow through and placental tissue, respectively. These LODs correspond to sample Ti concentrations of 0.11 µg/mL, 0.02 µg/membrane, 0.05-0.06 µg/mL and 3.00 µg/g tissue, respectively, when accounted for dilution (dotted lines in figures).

Statistics

The data in this study is presented as mean ± standard deviation (SD) of 3 biologically independent experiments (each representing e.g. a different cell passage or an individual placenta) with 1-3 technical replicates (same cell passage; replicates done within one experiment) unless otherwise stated. (Differences between viabilities of untreated cells vs. different treatment conditions were analyzed using an unpaired Student's t-test (GraphPad Software Prism 6, San Diego, CA, USA). The same statistical test was applied to compare *in vitro* TiO_2 NP distributions in the absence or presence of cells or between both monolayers, or each monolayer and the co-culture (e.g. BeWo vs. co-culture) as well as *ex vivo* uptake into placental tissue. Statistical significance was obtained when $p < 0.05$ (*).

Results

NP characterization

The zeta potential of TiO_2-NH_2 and TiO_2-COOH NPs in H_2O confirmed their surface modification showing a positive and negative value, respectively (table 1). When measured in EM and PM, a slightly negative zeta potential was determined for both TiO_2 NPs comparable to measurements of EM or PM only. Primary particle size of both TiO_2 NPs is approximately 4-8 nm (supplement fig. S1) but exact numbers could not be determined due to the poor contrast and overlap of individual particles. DLS measurements were done to investigate potential agglomeration of the NPs in the different experimental media over time. Indeed, the hydrodynamic diameter of TiO_2-NH_2 NPs in EM and PM decreased from 3482.0 ± 291.8 nm and 789.9 ± 120.5 nm at the beginning (0 h) to 292.5 ± 33.6 nm and 128.7 ± 32.23 nm after 24 h of incubation at 37 °C, respectively (fig. 1 and supplement table S1). This decline indicates that bigger NP agglomerates were formed in medium suspensions immediately after adding the particles, which then sedimented and disappeared from the detection window of the DLS. At time point 0 h, hydrodynamic diameters of TiO_2-COOH NPs were comparable in EM (1389.3 ± 57.6 nm) and PM (1532.3 ± 60.5 nm). Agglomerate sizes initially increased to 2308.0 ± 113.4 nm in EM after 6 h and 1826.5 ± 1077.4 nm in PM after 24 h demonstrating a slower agglomeration behavior of TiO_2-COOH NPs compared to TiO_2-NH_2 NPs. The PDI determined in EM and PM strongly varied for both particles over time, indicating highly polydisperse suspensions. In H_2O, both TiO_2 NP types were relatively stable with sizes varying only between 43.2 ± 12.6 nm and 78.4 ± 1.21 nm, and PDI values in the range of 0.16 to 0.21. Agglomeration and sedimentation of the TiO_2 NPs was confirmed by visual observation of particle suspensions that were incubated for 0 h, 6 h (maximum for *ex vivo* perfusions) and 24 h (maximum for *in vitro* translocation studies) at 37 °C/5% CO_2 in static conditions (supplement fig. S2). A white pellet was observed already after 6 h of incubation in both media and light microscopy showed the precipitation of NP agglomerates at the bottom of a well plate.

Table 1: Primary particle size and zeta potential of TiO_2-NH_2 and TiO_2-COOH NPs.

		TiO_2-NH_2	TiO_2-COOH
Primary size [nm][a]		4-8	
Zeta potential [mV][b]	in H_2O	56.20 ± 8.37	-37.83 ± 3.21
	in EM	-11.63 ± 0.53	-8.75 ± 0.53
	in PM	-13.20 ± 0.78	-5.97 ± 0.59

[a] Target size for core NPs according to the manufacturer

[b] Data are shown as mean ± SD.

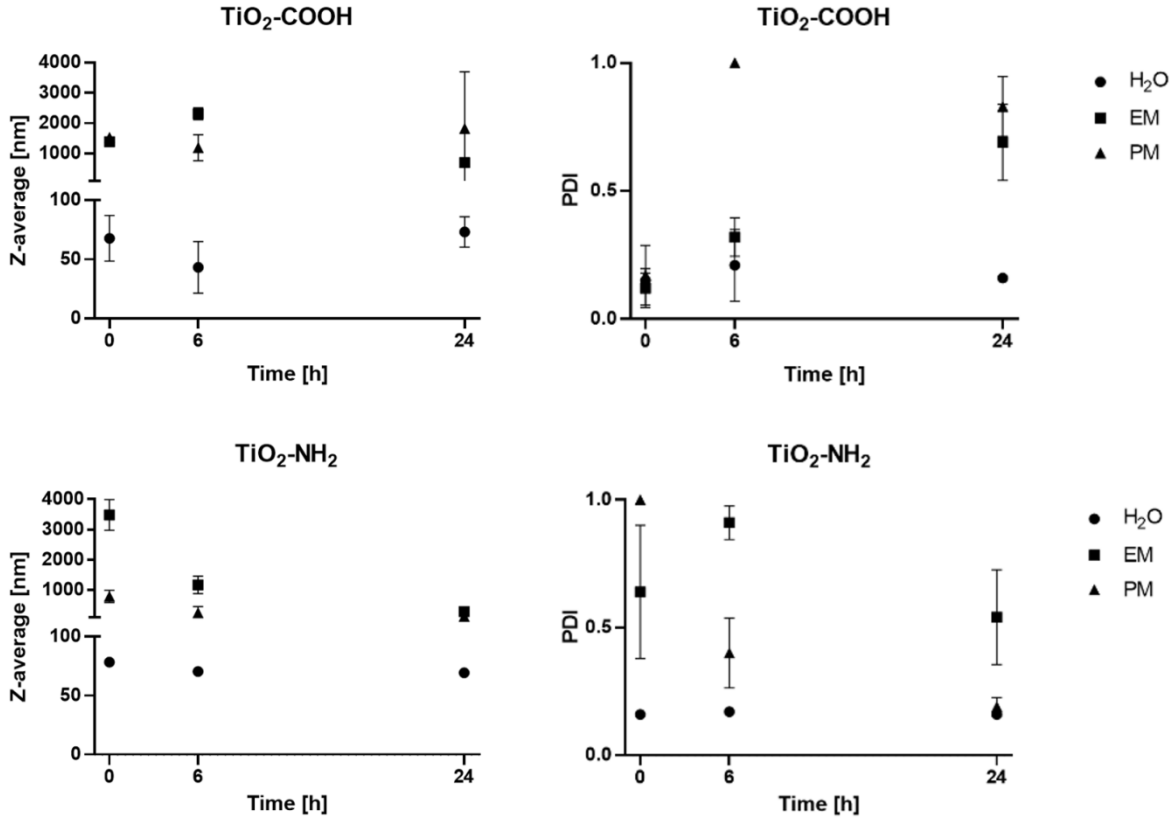


Fig. 1: Hydrodynamic diameter (Z-average) and PDI values of TiO_2-COOH and TiO_2-NH_2 NPs in $H_2$0, EM, and PM after 0, 6 and 24 h of incubation at 37 °C. Data are shown as mean ± SD from 3 measurements.

Impact of TiO_2-NH_2 and TiO_2-COOH on trophoblast viability

The effect of TiO_2 NPs on trophoblast and endothelial cell viability was investigated before the *in vitro* translocation study to exclude acute cell toxicity (fig. 2 and fig. S3). Both TiO_2 NP types did not elicit any considerable cytotoxic responses in BeWo and HPECs at concentrations selected for translocation studies.

After 6 h, treatment with 10 µg/mL TiO_2-NH_2 and TiO_2-COOH NPs, cell viabilities were 121.0 ± 17.0% and 109.0 ± 3.1% for BeWo cells and 127.6 ± 12.0% and 129.0 ± 5.3% for HPECs, respectively. Increased cell viability was also observed after treatment with 1 µg/mL TiO_2-NH_2 and TiO_2-COOH NPs for 24 h with 116.1 ± 6.3% and 109.5 ± 2.1% for BeWo and 112.4 ± 3.4% and 118.1 ± 3.8% for HPECs, respectively. In general, slight but significant effects on HPEC and BeWo cell viability (positive and negative) were observed occasionally but without a clear dose- and time-dependency.

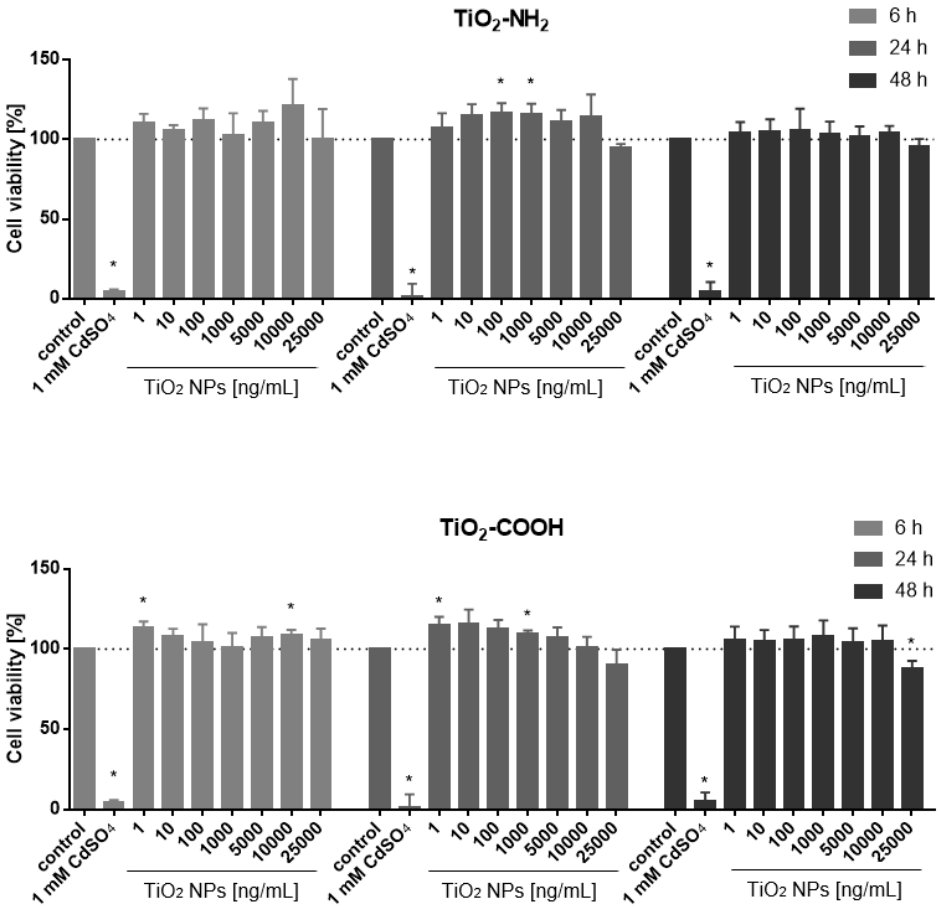


Fig. 2: BeWo cell viability after TiO_2-NH_2 and TiO_2-COOH NP treatment measured via MTS assay. BeWo cells were treated for 6 h, 24 h and 48 h with TiO_2-NP concentrations ranging from 0 to 25 µg/mL in EM. 1 mM $CdSO_4$ was used as positive control. The dotted line indicates 100 % viable cells. Data is shown as mean ± SD of 3 biologically independent experiments with 3 technical replicates each. * for $p \leq 0.05$

In vitro placental uptake and translocation of TiO_2-NH_2 and TiO_2-COOH

In vitro placental uptake and translocation was investigated using confluent monolayers of BeWo cells or HPEC-A2 cells, or co-cultivated membranes representing the two

key cell types (trophoblasts and microvasculature endothelial cells) of the human placental barrier at term. 1 µg/mL of each TiO_2 NP type (corresponding to 0.53 ± 0.05 Ti for TiO_2-NH_2 and 0.39 ± 0.06 µg/mL Ti for TiO_2-COOH NPs; fig. 3A) was added apically to the cell cultures, and the basolateral supernatant was sampled after 6 and 24 h whereas apical supernatants and the membranes were collected after 24 h.

SF-ICP-MS analysis revealed that all Ti concentrations obtained in the basolateral supernatants after 6 h were below the LOD and similar to background levels determined in the EM only (fig. 3A). After 24 h of exposure, the Ti content in the basal compartment were still below to the LOD. Translocation across the empty membrane was only slightly increased with 0.11 ± 0.09 µg/mL or 61.71 ± 46.55 % for TiO_2-NH_2 NPs. Also in the apical compartment, Ti concentrations were below or close to the LOD after 24 h of exposure, suggesting sedimentation of the particles.

The analysis of the membrane fractions revealed that both TiO_2 NP types were internalized by the different cells and/or adhered to their surface (fig. 3B). For TiO_2-NH_2 NPs, Ti concentrations of 0.15 ± 0.04 µg/membrane (57.33 ± 13.95 %), 0.14 ± 0.05 µg/membrane (51.24 ± 14.89 %) and 0.14 ± 0.03 µg/membrane (55.71 ± 17.55 %) originating from TiO_2-NH_2 NPs were detected in BeWo or HPEC monolayers and co-cultures, respectively. Uptake of TiO_2-COOH NPs was 0.08 ± 0.02 µg/membrane (41.63 ± 4.34 %) for BeWo monolayers, 0.06 ± 0.02 µg/membrane (27.39 ± 8.45 %) for HPEC monolayers and 0.07 ± 0.01 µg/membrane (34.56 ± 2.42 %) for co-cultures. Moreover, a notable interaction of TiO_2-NH_2 and TiO_2-COOH NPs with the collagen-coated membrane was detected with 0.06 ± 0.02 µg/membrane (21.33 ± 5.47 %; statistically significant) and 0.03 ± 0.03 µg/membrane (15.33 ± 11.87 %; not significant), respectively, when compared to membranes without NP treatment.

TEER values were determined before and after 24 h TiO_2 NP exposure to understand potential effects on barrier integrity (supplement fig. S4). While both TiO_2 NP did not alter TEER of HPEC monolayers, a slight but non-significant decrease was observed for BeWo monolayer and co-culture barriers.

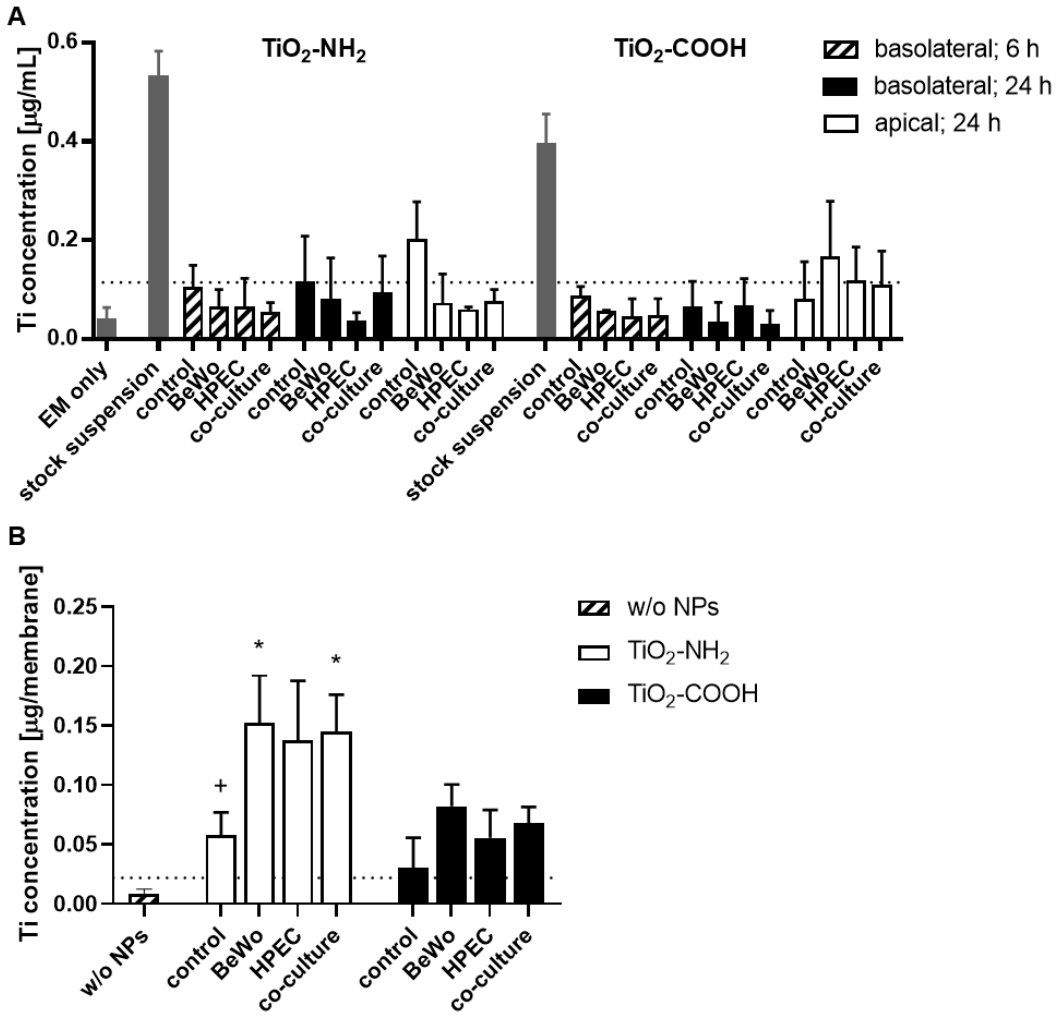


Fig. 3: Distribution of Ti *in vitro* after TiO_2 NP exposure. Cell monolayers and co-cultures were treated for 24 h with 1 µg/mL TiO_2-NH_2 or TiO_2-COOH NPs under static conditions. Afterwards, Ti contents were determined in the (A) apical, basolateral (6 h & 24 h) and (B) membrane fraction by SF-ICP-MS. Collagen coated membranes without (w/o NPs) and with NP exposure (control) were used to determine the intrinsic Ti content and the interaction between the TiO_2 NPs and the membrane, respectively. Data is presented as mean ± SD from 3 biologically independent experiments with one replicate each. The dotted line indicates the LOD when accounted for dilution of the samples during ICP-MS measurement. $^+$ and * (both $p \leq 0.05$) demonstrate statistical significance between the control condition and the membrane without NP treatment or the control condition and the mono-/ and co-cultures, respectively.

Ex vivo placental uptake and translocation of TiO_2-NH_2 and TiO_2-COOH NPs

Placental translocation and tissue uptake of both TiO_2 NPs (10 µg/mL corresponding to 3.28 ± 0.54 µg/mL Ti for TiO_2-NH_2 NPs and 3.41 ± 1.76 µg/mL Ti for TiO_2-COOH NPs) was also assessed in the dynamic *ex vivo* placenta perfusion model for up to 6 h (fig. 4). The maternal Ti concentration decreased for both TiO_2 NPs, however, with different kinetics (fig. 4A). While levels dropped from 3.28 ± 0.54 µg/mL at 0 h to 1.91 ± 0.50 µg/mL at 6 h of perfusion for TiO_2-COOH NPs, a rapid reductionof Ti from 3.41 ± 1.76 µg/mL at 0 h to 0.60 ± 0.24 µg/mL at 0.5 h and 0.10 ± 0.03 µg/mL at 6 h of perfusion for TiO_2-NH_2 NPs was observed. Despite a lower LOD for Ti in PM (0.05-0.06 µg/mL in PM vs. 0.11 µg/mL in EM when accounted for sample dilution), Ti

contents in the fetal flow through could not be clearly detected. For both TiO_2 NPs, the fetal Ti concentrations after 6 h of perfusion were below the LOD (0.03 ± 0.01 µg/mL for TiO_2-NH_2 NPs and 0.04 ± 0.02 µg/mL for TiO_2-COOH NPs). Only at some time points, did fetal Ti concentrations slightly exceed the LOD during the perfusion with the highest levels of 0.12 ± 0.10 µg/mL (4 h) and 0.06 ± 0.06 µg/mL (4 h) after TiO_2-NH_2 and TiO_2-COOH exposure, respectively.

Accumulation of Ti in placental tissue was observed after 6 h of perfusion with TiO_2-NH_2 NPs (161.70 ± 253.50 µg/g tissue) as well as TiO_2-COOH NPs (19.80 ± 8.95 µg/g tissue) (fig. 4B). However, large variations were obtained among individual perfusion experiments, most likely due to inhomogeneous distribution of particles in the perfused cotyledon (only a small piece of the perfused cotyledon was digested).

Control perfusions without placental tissue were performed to investigate potential interactions of the TiO_2 NPs to the experimental device, such as adsorption. Ti concentrations gradually declined from 4.51 µg/mL to 0.18 µg/mL for TiO_2-NH_2 NPs and from 3.09 µg/mL to 0.16 µg/mL for TiO_2-COOH NPs after 6 h of perfusion (fig. S5). Importantly, low Ti concentrations were also detected after the perfusion of the maternal side with PM only (fig. S5) and these values were in the same range as fetal Ti concentrations measured in perfusion experiments with TiO_2 NPs (fig. 4A). This indicates that a potential elution of Ti from the system (e.g. tubes) cannot be fully excluded.

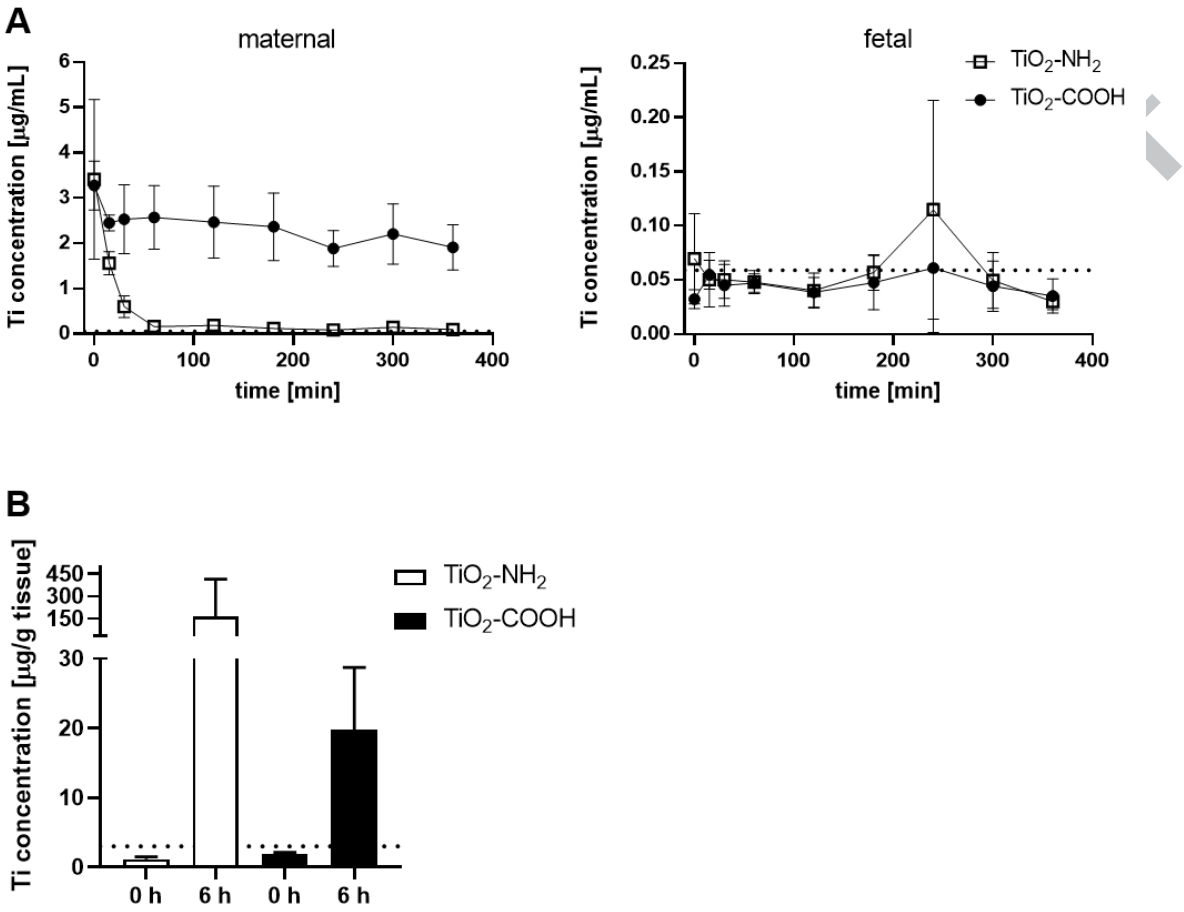


Fig. 4: Placental translocation and accumulation of TiO_2 NPs *ex vivo*. Human placental tissue was perfused with 10 µg/mL TiO_2-NH_2 or TiO_2-COOH NPs for 6 h. Ti contents were determined via SF-ICP-MS in maternal and fetal suspensions over time (A) and in tissue samples of each placenta before and after the perfusion (B). Data is shown as mean ± SD from 3 biologically independent experiments. The dotted line indicates the LOD when accounted for dilution of the samples during ICP-MS measurement.

Discussion

There is evidence that NP characteristics such as surface modification can be tailored to steer NP uptake and translocation at the placental barrier [18]. Here we investigated the uptake, accumulation and translocation of positively and negatively charged TiO_2 –COOH and TiO_2–NH_2 NPs at the human placental barrier. TiO_2 NPs were selected due to their widespread use in food and cosmetic products and the reported developmental toxicity in mice [19]. To study charge-dependent effects on placental distribution we used custom-synthesized model TiO_2 NPs with similar primary size functionalized with carboxylate or amine groups, which are not necessarily representative of food-relevant TiO_2 NPs. Another key consideration of biodistribution studies is the use of realistic exposure pathways and doses since these can profoundly

affect the study outcome [33,34]. Due to the use of TiO_2 NPs in consumer products, pregnant women are exposed most likely via the dermal or the oral route. Since it was shown that NPs do not cross the intact skin barrier [35,36], we considered oral exposure to TiO_2 NPs as the most relevant route for pregnant women. In the US, it was estimated that the dietary intake of TiO_2 is 0.2-0.7 mg/kg body weight per day for adults [37,38]. Considering that only around one third of TiO_2 particles in common food products are nano-sized and that transfer at the intestinal barrier is low (~0.1%) [39], systemic concentrations of a few ng/ml may be expected. However, such amounts are too low to be reliably detected in our models by ICP-MS, where we reached LODs of 0.2-0.6 ng/mL in supernatants and 5 ng/mL in tissue digests (LOD from undiluted samples). Moreover, we were facing difficulties with matrix effects and had to highly dilute our samples (200x dilution), which further prevented the use of low doses. Therefore, we chose a higher dose of 10 µg/ml NPs for the 6 h *ex vivo* placenta perfusions and 1 µg/mL for *in vitro* transfer studies due to the longer exposure duration (24 h). Nevertheless higher concentrations may be conceivable considering that TiO_2 NPs can accumulate in tissues over time and reach relatively high local concentrations due to their biopersistence [24,40,41]. Moreover, concentrations up to 100 µg/mL could be realistic for a potential biomedical use of TiO_2 NPs (e.g. injected intravenously as drug carrier) [42,43].

We did not observe any considerable translocation of amine or carboxyl-modified TiO_2 NPs to the fetal circulation after 6 h perfusion of human term placenta. Occasionally, a very low fraction of particles was observed in some fetal samples but these may not necessarily be from translocated particles since similar Ti background levels were also found in an empty perfusion with PM only. Even prolonged exposure for 24 h in an *in vitro* placenta co-culture transfer model did not reveal any particle transfer above the LOD of the ICP-MS analysis or the background Ti signal from the EM only. While TiO_2-COOH and TiO_2-NH_2 NPs did not cross the placental barrier, they were detected in the placental cell/tissue fraction. In principle, both BeWo cells and HPECs bind/internalize TiO_2 NPs but no additive effect was found in the co-cultures. Due to challenges to lyse the individual cell types of co-culture inserts in presence of a porous membrane, it was not possible to investigate which cell type bound/internalized more NPs in the co-cultures. Findings of the current study are in accordance with previous work, where no placental transfer but internalization of large uncoated TiO_2 NPs by trophoblast cells was found [44]. In addition, outcomes of previous studies demonstrated that large

particles or even graphene oxide sheets were internalized by placental cells/tissue [32,43]. However, we would like to note that during the current study we did not confirmed the uptake of the TiO_2 NPs via methods like e.g. TEM imaging.

In contrast, placental translocation to fetal brain and liver was detected by transmission electron microscopy or energy-dispersive X-ray spectroscopy after exposure of pregnant mice to TiO_2 NPs [19,22]. We cannot exclude that a very low amount of TiO_2 NPs, below the detection limit of the ICP-MS, did also cross the human placental barrier in our models but differences in placental structure and function among species or the use of different TiO_2 NPs may also account for the different outcomes in placental transfer among these studies.

In general, nonphagocytic cells internalize cationic NPs to a higher extent than anionic NPs [45]. Therefore, our finding that amine- and carboxyl-modification did not affect TiO_2 NP distribution at the placental barrier may be unexpected. However, since non-coated TiO_2 NPs did not pass the human placental barrier, it was not possible to detect a decrease in transfer as previously described for carboxyl-coated polystyrene NPs [46]. Higher placental translocation of more positively charged NPs has been observed in some studies (e.g. Fe_2O_3-PEI NPs [47]; Au-Ferritin NPs [48]) but not in others (Fe_3O_4-Oleate NPs [49]). We hypothesize that the low translocation of TiO_2-COOH and TiO_2–NH_2 NPs was due to the strong agglomeration of the particles in the biological media as evidenced by DLS measurements and visual observation. Consequently, size effects may mask more subtle charge-dependent effects. While both NP types were relatively stable in H_2O for 24 h, immediate agglomeration was observed for particles suspended in both biological media. Colloidal instability of NPs in protein- and electrolyte-rich cell culture media is frequently observed and is likely the result of molecule/protein adsorption or loss of surface functionality, resulting in the formation of particle agglomerates/aggregates (reviewed in [50]). These processes can ultimately determine the fate and effect of the NPs in biological systems [51,52].

Upon further incubation of the NP suspensions under experimental conditions, the hydrodynamic size of TiO_2-NH_2 NPs in the supernatant rapidly decreased due to the sedimentation of larger agglomerates. The mean hydrodynamic diameter of the TiO_2-COOH NPs did not drop in PM and initially increased in EM after 6 h of incubation. In general, we would like to note that DLS is not ideal for the analysis of highly polydisperse suspensions and that further assays should be included for definite particle size measurements [53]. However, we could detect changes in the size and

PDI values of the NPs in the different media over time confirming strong particle agglomeration.Besides placental accumulation and translocation we also assessed the effects of TiO_2-COOH and TiO_2–NH_2 NPs on trophoblast and endothelial cell viability.Both particle types did not show any cytotoxic response of BeWo b30 trophoblast cell and HPECs at concentrations up to 10 µg/mL and 24 h of treatment. Furthermore, the absence of fetal-maternal leakiness in the *ex vivo* placenta perfusions confirms that NPs did not interfere with placental barrier tightness in this model. However, a tendency towards a decreased barrier tightness of the BeWo trophoblast layer was observed in the static co-culture model for both TiO_2 NPs, possibly due to the higher deposition of agglomerated NPs under static compared to perfused conditions. Further studies using other methods to assess barrier integrity (e.g. fluorescein exclusion assay) would be needed to more clearly understand if TiO_2 NPs interfere with placental barrier tightness.

Finally, we observed considerable interferences of the TiO_2 NPs with the placental system devices. In empty perfusion studies without placental tissue, TiO_2-COOH and TiO_2–NH_2 NPs strongly bound to the components of the perfusion system, thereby reducing the bioavailable dose. Interestingly, in the presence of placental tissue, a stabilizing effect presumably from placental proteins was observed for TiO_2-COOH NPs as concentrations only slightly decreased in the maternal circulation. In contrast, the drop in maternal levels of TiO_2-NH_2 was accelerated compared to empty perfusion, most likely due to the rapid uptake of a considerable fraction of particles by the tissue. In the *in vitro* placental transfer model, interferences with the microporous membrane were evident such as particle adsorption to the membranes since almost all particles disappeared from the apical and basolateral supernatants. However, only a portion of the applied dose was recovered in the membranes probably due to the washing for TEER measurements before membranes were collected for ICP-MS analysis. Recovery was improved in the presence of cells since particles were attached to and/or internalized by the cells and not removed during the washing step.

Conclusions

TiO_2 NPs functionalized with positively (-NH_2) or negatively (-COOH) charged groups show placental distribution highly similar to uncoated particles. They did not translocate across the human placental barrier *ex vivo* and *in vitro* but accumulated in placental cells and tissue in considerable amounts. Characterization of colloidal stability of our NP suspensions over time revealed a fast and strong agglomeration of positively and

negatively charged TiO_2 NPs in biological media suggesting that particle size was the key factor that determined placental uptake and transfer. Although surface charge did not affect NP distribution in our study, it may become relevant if particle agglomeration could be reduced by further functionalization strategies. In general, colloidal stability of NPs in biological suspensions needs to be carefully evaluated in mechanistic transport studies to distinguish between size- and charge-dependent effects.

Future studies should further address the short- and long-term consequences of placental accumulation of TiO_2 NPs on placental tissue function and signaling to understand if these particles may induce fetotoxicity by indirect placenta-mediated mechanisms. Such indirect developmental toxicity of NPs has not yet been systematically addressed and warrants increasing attention [23].

Declarations

Ethics Approval and consent to participate

The use of human placental tissue during the *ex vivo* perfusions was performed following the principles of the Declaration of Helsinki and written informed consent was given by the expecting mothers before delivery. The study was approved by the local ethics committee (EKOS 10/078).

Availability of data and material

The numerical data used in this manuscript and the corresponding supplementary information is available at https://doi.org/10.5281/zenodo.2610196.

Competing interests

The authors declare that they have no competing interests.

Funding

This research is supported by funding from the BMBF-project NanoUmwelt (03X0150) and Swiss National Science Foundation (grant no 31003A_179337).

Author's contribution

LA and TB designed the study and prepared the original manuscript. LA conducted the *in vitro* experiments and analyzed the data. BBD and MH performed size and zeta potential measurements of the TiO_2 NP suspensions. LA, AW, and RS determined Ti concentrations via SF-ICP-MS. PM performed the *ex vivo* placenta perfusions. SS provided TEM images of the TiO_2 NPs. PW, MS and YK provided important intellectual input and all authors were engaged in revising and commenting on the manuscript.

Acknowledgment

The authors would like to thank Prof. G. Desoye (Department of Obstetrics and Gynecology, Medical University Graz, Graz, Austria (with permission from Prof. P. Friedl, Institute of Biochemistry, Technical University Darmstadt, Darmstadt, Germany)) and Prof. Dr. Ursula Graf-Hausner (Zurich University of Applied Science (with permission from Dr. Alan L. Schwartz, Washington University School of Medicine, MO, USA)) for providing HPEC-A2 cells (SV40-transformed microvascular human placental venous endothelial cells) and the human placental choriocarcinoma cell line BeWo b30, respectively. Furthermore, we would like to thank Lukas Widmer, Melanie Senn and Sandro Lehner (Empa, St. Gallen & Dübendorf, Switzerland) for their assistance in the *in vitro* viability assay and sample preparation for the SF-ICP-MS measurements. Finally, the authors would like to acknowledge the collaboration with Carsten Jost (PlasmaChem Berlin, Germany) regarding NP synthesis and supply

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