

Nanospecific Guidance in REACH: A Comparative Physical-Chemical Characterization of 15 Materials with Methodical Correlations

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Abstract

The REACH legislation deals with the Registration, Evaluation, Authorization and Restriction of Chemical substances in Europe. Here we provide a comparative physical-chemical characterization with standardized methods that adhere to the recent REACH guidance for nanomaterials. The suite of materials comprises two pigment-size reference materials, and 13 engineered nanomaterials, thereof four from the OECD sponsorship program. We report for each material on 20 endpoints for physical-chemical properties. Since toxicological effects and occupational exposures have already been reported for several of these materials, our results provide the basis for the exploration of grouping approaches. On the methodical level, our results provide a cross-validation where several methods report the same endpoint. This comparison reveals redundancies in the guidance and allows us to identify the most effective methods.

Keywords: Suspension, size distribution, dissolution, photocatalysis, regulation

1. Introduction

Many ceramic materials of sub-micron particle sizes have to be considered as nanomaterials for regulatory purposes as specified by the European nanodefinition recommendation¹. The acronym REACH (Registration, Evaluation, Authorization and Restriction of Chemical substances) designates the European Community regulation on chemicals and their safe use. The aim of REACH is to improve the protection of human health and the environment through the better and earlier identification of the intrinsic properties of chemical substances. Since standard methods developed for molecules may be insufficient for nanomaterials, an implementation project for nanomaterials has proposed changes to the REACH guidance documents². Additional physical-chemical properties require the validation of suitable methods and the condensation of their measurement results to single figures or “endpoints” for regulatory dossiers². Very recently, the European Chemicals Agency (ECHA) drafted a nanospecific guidance document, designated as Appendix R7-1³, which is compatible with the recommendations of the OECD sponsorship program for safety testing of manufactured nanomaterials^{4,5,6}. Here we propose an efficient realization of physical-chemical characterization, which we exemplify on commercially relevant materials: nanofillers (BaSO₄, SiO₂, mwCNT, Carbon Black), cosmetic UV pigments (ZnO, TiO₂), catalysts (CeO₂), functional ceramics (ZrO₂). Several of these are distributed as

part of the OECD sponsorship program; our investigation also reports on one of the few comparative characterization exercises.

Many consortia pursue comparative toxicology with the goal of correlating as-produced with as-tested and as-released properties, and further to correlate uptake and effects *in-vitro* and *in-vivo*. Detailed characterization of nanoparticles is essential to understand their toxic effects and to develop Nano-QSARs⁷. The materials for the present investigation represent part of the *in-vivo* inhalation testing program performed at BASF over the last few years. Results of inhalation studies and *in-vitro* testing with these materials were reported by NanoCare (BMBF, 2006–2009)^{13,9,8,11,10,12}. To ensure continuity, a subset of materials has undergone further *in-vitro* and oral testing in the follow-up project nanoGEM (BMBF, 2010–2013) and are due to be reported independently. In order to allow for comparative toxicological assessments, the original batches were re-investigated according to the latest R7–1 guidance draft³. From the list of physical chemical endpoints that were specified by OECD and/or ECHA, the following properties with a total of 20 endpoints are reported here:

- Agglomeration/aggregation (R.7.1.14 in the guidance draft of 2012)
- Solubility in water (R.7.1.7) and in relevant media (R7.1.1).
- Dispersability (R7.1.1)
- Crystalline phase
- Crystallite size

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- Shape: representative electron microscopy image(s) (R.7.1.19)
- Particle size distribution – dry and in relevant media (R.7.1.14)
- Specific surface area (R.7.1.20)
- Zeta potential/surface charge
- Surface chemistry
- Photocatalytic activity
- Porosity

Our key result is a large table of (15 materials x 20 endpoints) that will be utilized by future research: Along a line (endpoint), one can compare materials and correlate them to hazard information. Along a column (material), the disagreement between methods identifies materials that deviate from generic particulates. Our realization of the characterization dossier suggests a testing strategy in order to streamline characterization based on the fact that some endpoints originate from the same measurement, such as the set of size distribution dry, aggregation, SEM image or size distribution in relevant media, agglomeration, dispersability, and some link to the same physical measure (porosity, specific surface area) – whereas some are redundant and may be re-considered in future revisions to the guidance.

In the present contribution, we do not address the deviation from established exposure thresholds in mass metrics to a nanodefinition in number metrics¹; the interested reader is referred to several independent investigations into the validity of granulometry methods for number-based identification purposes.^{16, 14, 15}

II. Materials

The main purpose of the present contribution is the comparative characterization of materials. The present section is hence restricted to the commercial sources and applications, generic synthesis route and identifying synonyms in various projects. Among the materials reported, four are available from the OECD nanomaterial repository (NM 105, NM111, NM220, NM 400), seven are available commercially, four were NanoCare-internal materials. All of them have received extensive toxicological testing in the NanoCare project^{13, 9, 8, 10, 12, 17}.

TiO₂: The coated TiO₂ (tradename T-Lite™ SF) was commercialized by BASF for cosmetics applications as a UV-protective agent. It is produced by means of wet synthesis and is intended to carry an aluminium hydroxide coating in order to passivate the TiO₂ surface, and further topped by a dimethicone/methicone copolymer coating in order to stabilize it in sunscreen formulations. The ecotoxicity has been reported earlier, in comparison to other TiO₂ and ZnO materials¹⁸. Here we compare the physical-chemical properties of the coated TiO₂ against the widely studied TiO₂ NM105 (designated as “TiO2_3” in ref.¹³ and “TiO2_B” in ref.⁸), which is synthesized by means of pyrolysis and has no coating.

ZnO: Particles from ZnO are used as UV absorbers. Two materials, a coated nano-scale material (ZnO coated = OECD NM111), and a micron-scale, uncoated material were tested in parallel. The triethoxycaprylyl-silane polymer coating is applied after flame spray pyrolysis and is intended to stabilize the ZnO in the oily phase of a cos-

metics product by hydrophobization of the particle's surface. The ecotoxicity has been reported earlier, in comparison to other TiO₂ and ZnO materials¹⁸, and the physical-chemical properties have been studied in detail in the PROSPECT project¹⁹.

Carbon black (CB) is a material produced by the incomplete combustion of petroleum products (e.g. coal tar) or vegetable oil. CB is used as a black pigment and reinforcement in rubber and plastic products. The specific CB used here is a semi-active carbon black from pyrolysis with high structure and ash content of 0.5 %, and is used as mechanical reinforcement in rubber: tire carcass, tube, belt.

Silicas are distinguished into two major forms: crystalline and amorphous silica. Quartz, as a micron-size crystalline compound, induces silicosis and possibly cancer in humans²⁰. In contrast, the biological effects of synthetic amorphous silicas (SAS) appear to be transient²¹. SAS are frequently used as filling materials in plastics, lacquers, paints, tire compounds, but also in tooth pastes, pharmaceuticals, cosmetics, and foods. SAS can be subdivided into wet-process-manufactured (precipitated) silica, pyrogenic silica and surface-treated silica. In this study we used a precipitated product (identical to nanoGEM.SiO₂.naked), and a surface modification thereof with a covalently attached Polymethacrylic acid/3-Methacryloxypropyl copolymer (BASF research) that provides negatively charged electrosteric stabilization.

Multi-Wall Carbon Nanotubes MWCNT may serve many upcoming applications in the electronics, energy, construction and aerospace industries as they offer unique electrical, mechanical, and thermal properties. MWCNT have been reported to induce inflammation and may lead to granuloma formation after inhalation²². The morphology is thought to be decisive, and hence two MWCNTs of different manufacturers, but all based on catalyzed vapor deposition, were compared to the OECD batch CNT NM400.

BaSO₄: Nanoscale barium sulfate is used as a filling material in polymer compositions to increase scratch resistance while conserving transparency. The material (same product as NM220) is milled down to the below-micron range and was benign in preliminary short-term inhalation testing⁸ and in *in-vitro* assays¹⁷. BaSO₄ generally shows little biological activity and has been used for a long time as a contrasting material in medical diagnosis based on X-ray applications.

ZrO₂: Industrial applications include foundry sands, refractories, and ceramics. The material is also used as coating material for hip joint endoprostheses, or dental prostheses. ZrO₂ particles increase durability and scratch resistance of enamels and varnishes. Micron-scale ZrO₂ infers a comparatively low carcinogenic potential under severe overload conditions²³. The specific ZrO₂ investigated here was a pyrolytic research product (BASF) for NanoCare. Little comparative data is available on the effects of nano-scale ZrO₂ particles in the lungs⁸ and the nanoGEM project will report on several ZrO₂ surface modifications.

CeO₂: Nanoparticles from CeO₂ are widely used e.g. in solar cells, as additives in diesel and automotive catalyt-

ic converters, as ultraviolet absorbents and also for glass or ceramic applications. Both oxidative/toxic and anti-oxidative/protective effects on tissue have been described for CeO_2 ²⁴, which relates to the coexistence of Ce^{3+} and Ce^{4+} in crystalline edges of nanoparticulate material²⁵. In this investigation we tested two non-commercial types of CeO_2 particles which partly differed in chemical composition as one was pure (designated as “ CeO_2 ” in ref.¹³) while the other was doped with a low percentage of aluminium, both made by means of pyrolysis within the NanoCare project.

III. Methods

a) *Size, shape, aspect ratio, representative image, agglomeration, size distribution – dry: electron microscopy*

The primary NPs size and shape were assessed using a transmission electron microscope (TEM) from FEI, Type Strata 400 DB, equipped with a field emission cathode. For TEM analysis, samples were wetted in ethanol, then gently spread on a sample holder and transferred into vacuum for TEM imaging. For agglomerates of the powder materials, a field of view exceeding $10\ \mu\text{m}$ is required, and hence SEM is the only option spanning the entire size range down to smallest agglomerates of few primary particles. The powders were transferred onto an adhesive pad of a SEM sample holder and coated with a thin electrically conducting Pt layer via Ar ion sputtering (Baltec SCD 500). The samples were imaged in a SEM (JSM 7500 TFE, Jeol or Strata 400, FEI) both equipped with a field emission gun using secondary electrons and an acceleration voltage of typically 5 kV. The data analysis is analogous to TEM.

b) *Crystalline phase, crystallite size: X-ray Diffraction (XRD)*

Crystallite size and crystalline phase were determined by means of standard XRD. The sample is filled into the sample holder as delivered. In the case of powders, the surface is flattened using a glass plate. The Bruker D8 Advance Series II Diffractometer acquires the intensity as function of the diffraction angle with the Sol-X detector and variable slits (V20). The range of $2^\circ < 2\theta < 80^\circ$ is scanned in step width $0.02^\circ (2\theta)$ in intervals of 2.4 s. The crystallite size determination was performed using line profile analysis. The fundamental parameter approach²⁶ as implemented in the software package TOPAS²⁷ was used for this determination. A Lorentzian crystallite size contribution to the total line profile was assumed²⁸.

c) *Specific surface area*

Specific surface area was determined with the BET method on 50- to 300-mg samples, depending on the total pore volume in the measuring range of 0.1 to $1000\ \text{m}^2/\text{g}$. Samples were decontaminated at 200°C under vacuum. Nitrogen adsorption/desorption isotherms at 77 K were recorded at five pressures between 0 and 0.2 P/P₀. The measurements were distributed between different instruments – Autosorb 6b (Quantachrome) or Tristar or ASAP2420 (both Micromeritics) – all adhering to the standard DIN 66131.

d) *Agglomeration, Porosity: Hg-intrusion*

Mercury porosimetry was performed and evaluated according to DIN 66133 (analogous to ISO 15901 Part 1). Between 0.01 and 0.5 g of powder sample are required depending on the total pore volume. The samples are gradually intruded at pressures up to $p = 4000\ \text{bar}$ while the incremental volume change dV is recorded in the AutoPore IV (Micromeritics). The integration of the $p\ dV$ curve provides the specific surface²⁹. The pressure required to force the liquid into a pore is directly related to the inner diameter of the pores (assumed to be of cylindrical shape)³⁰, since the surface tension of Hg is known and its contact angle of little practical variation. All measurements are performed at room temperature. To prevent drifts, lab-internal standards are measured in regular intervals, but absolute calibration relies on the manufacturer. The instrument switches from negative to positive pressures at $7.5\ \mu\text{m}$ pore size, often resulting in an artificial dip in the pore size distribution.

e) *Surface chemistry: X-ray Photoelectron Spectroscopy (XPS)*

The chemical composition of the material's surface and the intentional or inadvertent organic surface modification were determined with a PHI XPS 5500 system equipped with 300 W monochromatic Al $K\alpha$ radiation. The pass energy for surveys was 117 eV (measurement time of 45 min) and for detailed spectra 23.5 eV (measurement time of 6 min). In this case, spectra evaluation was performed by CasaXPS 2.3.15, based on the PHI standard-sensitivity factors, with Shirley background subtraction and peak shape fits as sum of 90 % Gaussian and 10 % Lorentzian. Information depth of XPS is specific to the surface 1 nm to 10 nm of flat bulk materials, depending on cross-sections of photoelectron reabsorption. Owing to increased path lengths in rough surfaces, the XPS signal is even more specific to the surface for powders. Two measurements per sample were performed, each integrating over $0.5\ \text{mm}^2$. The results in % are derived from the relative concentration of elements and their chemical bonds from line shape analyses especially of the C(1s) photoelectrons.

f) *Photocatalytic activity: Methylene blue degradation*

The method closely follows DIN 52980:2008–10. The materials were dispersed at $c = 5\ \text{mg/l}$ concentration in an aqueous solution containing methylene blue, which has the known extinction coefficient $\epsilon = 7.402\ \text{m}^2/\text{mol}$. Before irradiation, the 5 mg/l dye solution has an optical absorbance of $\Delta A = 0.92$ at 664 nm wavelength. The effective particulate surface is given by $c \cdot \text{BET}$ and is in the range of $10^{-4}\ \text{m}^2/\text{ml}$ for typical nanomaterials. Beakers of 20 ml suspension were placed in the irradiation chamber (MRC Systems GmbH) which maintained a constant UV intensity of $E = 0.1\ \text{mW}/\text{cm}^2$ at $\lambda = 365\ \text{nm}$. Under constant stirring, the remaining dye absorption ΔA at $\Delta t = 0\ \text{h} - 2\ \text{h} - 6\ \text{h} - 22\ \text{h}$ was measured on aliquots in $d = 1\ \text{cm}$ thick cuvettes outside of the irradiation chamber. Suspensions of identical composition but kept in dark beakers defined the negative control ΔA_{dark} at the same time intervals. Following

the DIN standard, we calculate the unit-less photon efficiency ξ

$$\xi_{\text{MB}} = \frac{1}{\varepsilon \cdot d \cdot \text{BET} \cdot 30074 \cdot \delta \cdot E} \frac{\Delta A_{\text{dark}} - \Delta A}{\Delta t} \quad (1)$$

As further negative control, a dye solution without nanoparticles was UV-irradiated and showed no measurable degradation over 22 h.

g) *Surface charge: electrophoretic mobility, zeta potential at pH 7, isoelectric point*

The zeta potential was determined by means of Laser Doppler Electrophoresis using Zetasizer Nano (Malvern) and a folded capillary cell (DTS 1060, Malvern). The samples were dispersed at the room temperature in the background electrolyte solution (10 mmol/l KCl) and adjusted to the corresponding pH with 0.1 M NaOH or HCl. The concentration of the sample was adjusted to the signal intensity in the range of 3000–7000 kcps. The results are the average of the two measurements. The instrument was calibrated with Malvern Standard DTS 1235. We record the electrophoretic mobility across the titration range of pH 3 to pH 10, and report the iso-electric point as the pH at which the electrophoretic mobility crosses zero. Using the dispersed size and mobility and pH 7, we extract the zeta-potential at pH 7.

h) *Dispersability, size distribution in relevant media: Analytical ultracentrifugation (AUC) and Laser diffraction (LD)*

An interference-AUC (Beckman type XLI) with integrated interference optics was used, as described in detail earlier^{11,15}. Analogous to the less versatile, but standardized (ISO13318) and more widespread Centrifugal Liquid Sedimentation (CLS) technique, AUC relies on “detection during fraction”. (Planken and Colfen 2010) The samples were diluted to obtain concentrations of 1 mg/ml. Speed ramps from 1000 to 20 000 rpm were used to ensure complete coverage of the relevant measurement interval from 1 μm down to primary particles. The actual particle concentration is read directly from the optical fringe shift without any necessity for Mie conversion or other correction. The size distribution is evaluated with the freeware program Sedfit, with fitting models $g(s^*)$.

The larger agglomerates were determined with laser diffraction (Malvern Mastersizer 3000) in the range 0.5 μm to 500 μm , where optical constants have little effect on the resulting size distributions.

The average agglomeration number (AAN) was defined as the ratio of the volume-based median particle size (D50) to the primary particle size³.

i) *Solubility in water and relevant media: ICP-MS of supernatants (Inductively coupled plasma – mass spectrometry)*

Solubility methods that were established for micron-scale fibers are not generally applicable to nanomaterials, because these cannot be safely confined with standard filters. OECD methods that were established for classical chemicals are not applicable to nanomaterials³¹. Following earlier work on minerals, we assess the solubility with a

protocol that separates remaining particulate content from the ion solution by means of ultracentrifugation, one of the recommended protocols in a recent review³². The material is suspended in 20 ml water or DMEM/FCS at 10 mg/ml for a fixed dissolution time of 24 h at 25 °C. DMEM is Dulbecco's modified Eagle medium, low glucose, no pH indicators and was mixed with 10 % FCS (FBS Gold, defined Foetal Bovine Serum, PAA Laboratories). The ensuing ultracentrifugation at 300 000 g (in units of the gravitational acceleration $g = 9.81 \text{ m/s}^2$) for 10 h removes particles quantitatively down to about 2 nm diameter, as verified by AUC of the remaining particulate content in the supernatant. The content of metal ions in the supernatants was analysed by means of ICP-MS (Agilent 7500a) or ICP-AES (Varian 720). Samples are introduced via a Scott type spray chamber with a Meinhardt nebulizer (plasma power 1300 W, plasma gas 16.5 l/min, auxiliary gas 1.5 l/min). As internal standards, ⁴⁵Sc (ICP-MS) and Ar at 420.07 nm (ICP-AES) were used. 1 g sample was weighed in and diluted with 5 % (v/v) HCl to a total volume of 10 ml. Calibration standards were measured at 0 mg/l and 10 mg/l. The results in Table 1, last row, are given as the measured ppm levels of metal ions in the supernatant, which correspond to dissolution of 0.01 % of the dispersed material. The limit of detection was 0.1 ppm, corresponding to 0.001 % dissolution.

j) *Degradation (abiotic)*

As recognized by the OECD, any degradation phenomena need to be addressed by directly measuring degradation of the particles. The materials were dispersed at 2 mg/ml and kept at 37 °C in the dark under constant slow stirring at 100 rpm in relatively large suspension volumes of 20 ml in glass vessels with closed lids in order to reduce evaporation effects. After incubation, the degree of agglomeration was measured without further preparation by means of analytical ultracentrifugation (size range 1 nm to 3 μm , see above) and laser diffraction (Malvern Mastersizer 3000, size range 0.5 μm to 500 μm). On an independent aliquot, we ensured by means of hard centrifugation as above that nanoparticles down to 2 nm diameter were removed, and quantified the content of released ions with ICP-MS. Changes in the particle morphology were assessed with electron microscopy (TEM, SEM) and the crystallinity of samples was re-analysed with selected area electron diffraction (SAD).

k) *Incubation media for in situ testing*

The as-tested (*in-situ*) dispersability and solubility were tested after the materials had been stirred at 900 rpm for 24 h in DMEM (Dulbecco's modified Eagle medium, low glucose, no pH indicators) with 10 % FCS (FBS Gold, defined Foetal Bovine Serum, PAA Laboratories).

The as-tested (*in situ*) degradation was simulated by incubation for 28 d in phosphate-buffered saline (PBS, to simulate the surface of epithelium) or 28 d in phagolysosomal simulant fluid (PSF, to simulate the lysosome of macrophages)³³, or 1 d in 0.1 n HCl (to simulate oral uptake into the stomach) or 7 d in a Simulated Intestinal Fluid (fasted state, FaSSIF)³⁴. All incubation times were chosen

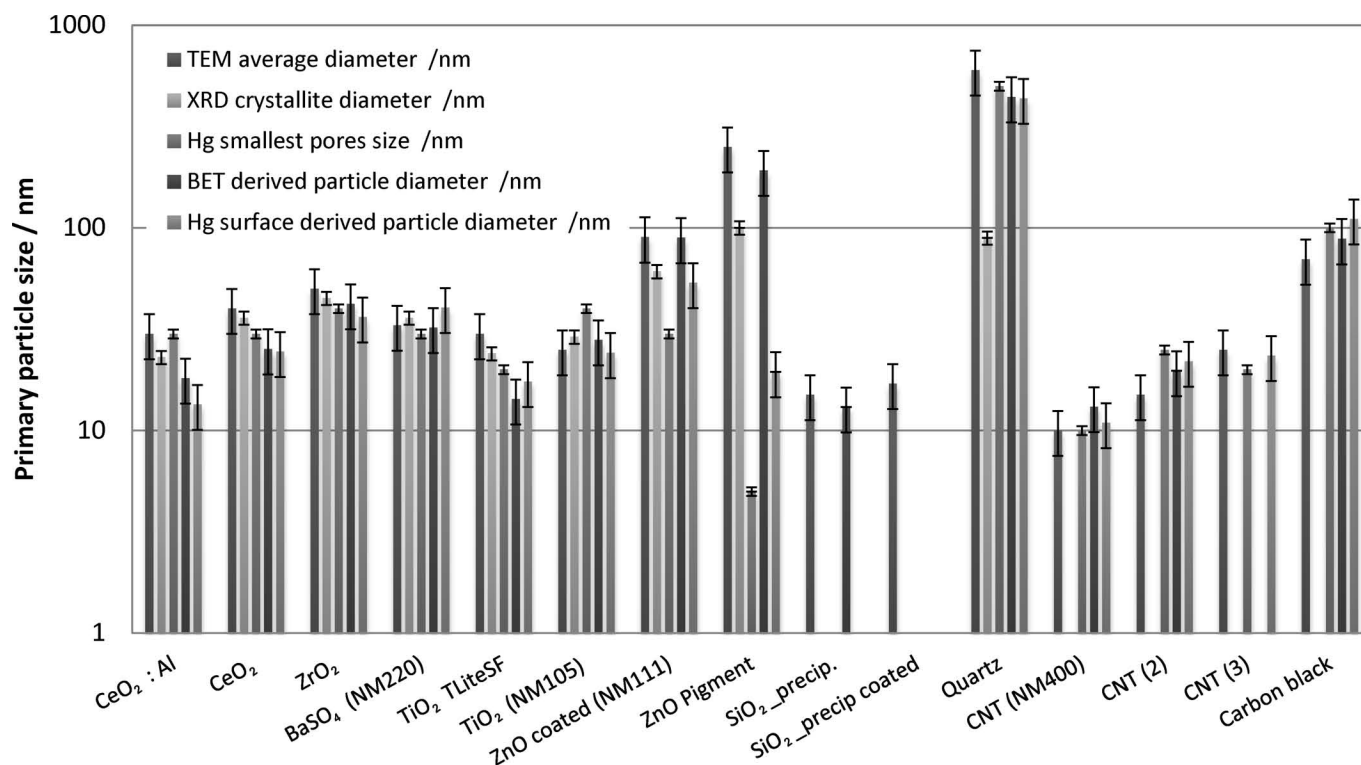


Fig. 1: Primary particle size. Diameters extracted from five methods for all materials tested.

at or above the maximum realistic residence time of nanomaterials in the specific body compartment, since we expected only weak dissolution effects.

The physiologic PBS composition (mg/l) with pH 7.4 is: 8 g/l NaCl, 0.2 g/l KCl, 1.44 g/l Na_2HPO_4 , 0.24 g/l KH_2PO_4 . The PSF composition followed the previous validation in ref.³³: Sodium phosphate dibasic anhydrous (Na_2HPO_4) 142.0 mg/l; Sodium chloride (NaCl) 6650 mg/l; Sodium sulfate anhydrous (Na_2SO_4) 71 mg/l; Calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) 29 mg/l; Glycine ($\text{C}_2\text{H}_5\text{NO}_2$) 450 mg/l (as representative of organic acids); Potassium hydrogen phthalate (1-(HO_2C)-2-(CO_2K)- C_6H_4) 4085 mg/l; Alkylbenzyltrimethylammonium chloride (ABDC) 50 ppm (added as an antifungal agent.). For incubation in PSF, the pH was adjusted to 4.5 by weekly addition of 0.11 ml of H_2O_2 (30 %) analogous to previous studies³⁵.

FaSSiF is a patented complex of taurocholate and lecithin, and validated for use of drug dissolution³⁴. We prepared the medium by dissolving 0.336 g of the Phares SIF powder (biorelevant.com) in 150 ml of a phosphate buffer (0.21 g NaOH, 1.977 g NaH_2PO_4 , 3.09 g NaCl in 500 ml H_2O), added in two steps.

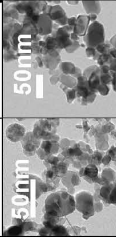
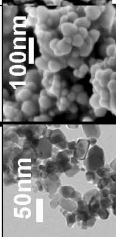
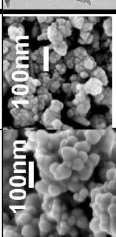
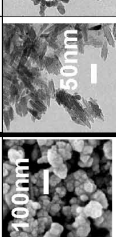
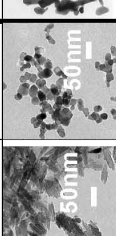
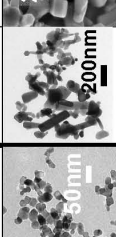
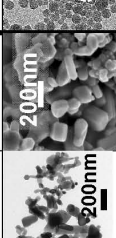
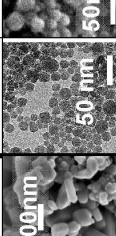
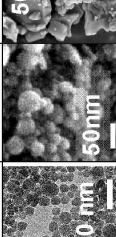
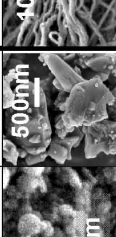
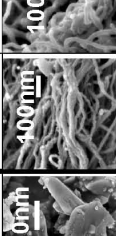
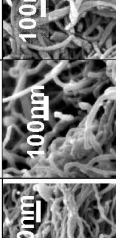
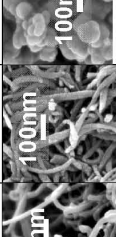
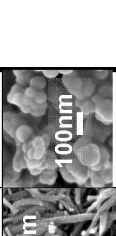
IV. Results

All measured values, sorted by regulatory endpoints, are summarized for all materials in Table 1, and additional findings on the persistence of selected materials are provided in Table 2. We translate the condensed numbers into words on the example of TiO_2 NM105, column six: The primary particles of TiO_2 NM105 have globular shape (embedded TEM image in Table 1) with 25 nm (up to 50 nm) diameter (Fig. 1) as determined with TEM.

In the as-produced powder, SEM detects typical agglomerate diameters (Fig. 2) of 20 000 nm. The crystallite size of 29 nm (from XRD line width) confirms that each primary particle is one crystallite. The crystalline phase is a mixture of anatase and rutile. Two peaks in the pore size distribution at 40 nm and 40 000 nm are found with Hg-intrusion (Fig. 2b), where the dip at 7 500 nm is instrument-related. The integrated mass-specific surface area of 59 m^2/g from mercury intrusion is close to the value of 51 m^2/g determined by means of BET nitrogen adsorption. The particle surface has an atomic composition of 20 % Ti, 56 % O, 23 % C, 1 % Zr (from XPS). These values need to be weighted by the molar masses to derive the mass composition of 43 % Ti, 40 % O, 12 % C, 4 % Zr. The photocatalytic activity that is expected for mixed rutile/anatase is clearly observable (Fig. 3a) with a photon efficiency of $2.4 \cdot 10^{-3}$.

When dispersed in water, TiO_2 NM105 crosses the IEP at pH 6.1, such that at pH 7 it has a low negative electrophoretic mobility of $-0.9 \mu\text{m/s/V/cm}$ and a derived zeta-potential of -12 mV at pH 7. The material forms large agglomerates of 2 700 nm in water, corresponding to an average agglomeration number³ (AAN) of 68. Less than 0.1 wt% of the dispersed material remains at diameters below 100 nm. In the popular cell culture medium DMEM with 10 % serum (FCS) the AAN is slightly reduced to 50 with a measurable content of 1 wt% of the material dispersed to diameters below 100 nm. All results presented so far are in close agreement with earlier characterization by independent labs in the NanoCare project¹⁷, thus serving as internal benchmark.

Table 1: Summary of physical-chemical endpoints for all measured materials.

	CeO ₂ : Al	CeO ₂	ZrO ₂ pyrolytic	BaSO ₄ (NM220)	TiO ₂ T-Lite SF	TiO ₂ (NM105)	ZnO coated (NM111)	ZnO Pigment	SiO ₂ precip.	SiO ₂ precip. coated	Quartz DQ12	CNT (NM400)	CNT (2)	CNT (3)	Carbon black
Representative image															
Particle size Distribution and Shape TEM: primary part. diameter / shape SEM: agglomerate diameter	2-160nm globular >20 000nm	10-200nm globular >10 000nm	25-60nm globular 1 000nm to 5 000nm	25nm globular 2 800nm to 15 000nm sphere	15 x 50 nm Aspect ratio >3, fiber	25-50nm globular >20 000nm	20-200nm mostly globular >20 000nm	50 – 500nm globular 2 500nm	8-25nm spherical n/a	10-30nm spherical n/a	100-7 000nm platelets 3 000nm	10nm fiber shape ~10 000nm bundles	15nm fiber shape ~20 000nm bundles	25nm fiber shape ~500 000nm pellets	50-100nm globular >20 000nm
Crystallite size (XRD)	23 nm	36 nm	45 nm	36 nm	24 nm	29 nm	61 nm	>100nm	n/a	n/a	89 nm	n/a	n/a	n/a	n/a
Crystalline phase (XRD)	Cerianite cubic	Cerianite cubic	ZrO ₂ tetragonal	Barite orthorhombic	Rutile, (minimally anatase)	Anatase / rutile	Zincite, ZnO hexagonal	Zincite, ZnO hexagonal	amorph	amorph	Quartz, SiO ₂ hexagonal	Graphite, C hexagonal with modified lattice const.	Graphite, C hexagonal with modified lattice const.	Graphite, C hexagonal with modified lattice const.	Graphite, C hexagonal with modified lattice const.
Pore sizes (Hg porosimetry)	30nm, 800nm, 40 000nm	30nm, 20 000nm	40nm, 1 000nm, 15 000nm	30nm, 5 000nm	20nm, 30 000nm	40nm, 40 000nm	30nm, 40 000nm	5nm, 200nm, 7 000nm	n/a (suspension)	n/a (suspension)	500nm, 30 000nm	10nm, 8000nm, 10 0000nm	25nm, 400nm, 150 000nm	20nm, 200nm, 20 000nm	100nm, 15 000nm
Specific surface area (BET and (Hg))	46.0 m ² /g (62)	33.0 m ² /g (34)	24.9 m ² /g (29)	41.4 m ² /g (33)	100.0 m ² /g (82)	51.1 m ² /g (59)	12.0 m ² /g (20)	5.6 m ² /g (55)	200 m ² /g nominally	n/a (suspension)	5.9 m ² /g (6)	250 to 300 m ² /g (324)	179 m ² /g (161)	n/d (151)	40.0 m ² /g (32)

	CeO ₂ : Al	CeO ₂	ZrO ₂ pyrolytic	BaSO ₄ (NM220)	TiO ₂ T-Lite SF	TiO ₂ (NM105)	ZnO coated (NM111)	ZnO Pigment	SiO ₂ precip.	SiO ₂ precip. coated	Quartz DQ12	CNT (NIM400)	CNT (2)	CNT (3)	Carbon black
Surface chemistry (XPS)	Ce 21 Al 9 O 56 C 9 Zr 4 N 1	Ce 16 O 61 C 9 (C-C, C-O, O-C=O) Al 9 Zr 5	Zr 24 O 53 C 19 (C-C, C-O, O-C=O) N 3 Al 1	Ba 13 O 52 C 17 S 11 Cl 3 P 3 N 1	Ti 16 O 63 C 9 Al 7 Si 5 Na <1 dimethicone/methicone copolymer as surface coating	Ti 20 O 56 C 23 Zr 1	Zn 1 C 64 (C-C, C-O, peptide) N 12 Na 2 P 2 Cl 1 3.5 % triethoxyoctyl silane	n/d	Si 29 O 66 C 4 (C-C, C-H, C-O, C=O) Na 1	Si 21 O 54 C 24 (C-C, some C-O, C=O) Na 1 Polymethacrylic acid / 3-Methacryloxypropyl	n/d	C 99 O 1 Al <1 Si <<1	C 99 O 1 Si <<1	C 100 O <1 Si <<1	C 98 O 1 S 1 Cl <<1
Photocatalysis: photon efficiency	8.8E-04	1.6E-03	7.0E-04	1.1E-03	5.3E-04	2.4E-03	Not measurable (hydrophob)	5.0E-01	1.7E-5	5.9E-5	3.4E-03	Not measurable (hydrophob)	Not measurable (hydrophob)	Not measurable (agglom.)	Not measurable (hydrophob)
Surface charge IEP	8.5	7.5	6.5	3.3	6.5	6.1	Not measurable (agglom.)	Not measurable (agglom.)	<3.5	<3.5	<3.5	Not measurable (agglom.)	Not measurable (agglom.)	Not measurable (agglom.)	Not measurable (agglom.)
Mobility at pH 7	1.4	0.5	-0.9	-2.2	-0.2	-0.9			-3	-3.7	-4.0				
ζ-pot at pH 7	18 mV	6 mV	-12 mV	-28 mV	-3 mV	-12 mV			-38 mV	-47 mV	-51 mV				
Dispersability in water (AUC)	603nm	90nm	1 500nm	116nm	33nm	2 700nm	Agglom.	5 100nm	20nm	23nm	2 900	Agglom.	Agglom.	Agglom.	1 920nm
Av. Agglom. Number AAN	20	3.0	38	4.6	1.7	68	<0.01wt%	20	1	1	1	<0.01wt%	<0.01wt%	<0.01wt%	29
in DMEM/FCS							below 100nm	below 100nm	below 100nm	below 100nm	below 100nm	below 100nm	below 100nm	below 100nm	below 100nm
D50	94nm	54nm	87nm	285nm	3 900nm	>>2μm	550nm	950	420nm	26nm	3 950nm	77 000nm	53 000nm	31 000	67
Av. Agglom. Number AAN	3.1	1.8	2.2	11	195	>>50	18	3.8	16	1	1	7 700	3 500	1 240	1
Solubility in water and in DMEM/FCS	Ce, Al below 0.1 ppm	Ce below 0.1ppm	Zr below 0.1ppm	Ba 6 ppm	Ti 5 ppm	Ti < 0.1 ppm	Soluble at pH< 6	Zn <5 ppm Zn 50 ppm	Si 3 ppm	Si 28 ppm	Si 41 ppm	n/d	n/d	n/d	n/d

Table 2: Dispersability, dissolution, agglomeration in some relevant media for a selected subset of materials.

	PBS_28d	PBS_28d	PBS_28d	PBS_28d	PSF_28d	PSF_28d	PSF_28d	PSF_28d	FaSSIF_7d	FaSSIF_7d	FaSSIF_7d	HCl_1d	HCl_1d	HCl_1d	HCl_1d
	Size / μm	Fine fraction / wt%	Dissolution / wt%	Charge zetapot / mV	Size / μm	Fine fraction / wt%	Charge zetapot / mV	Dissolution / wt%	Size / μm	Fine fraction / wt%	Charge zetapot / mV	Size / μm	Fine fraction / wt%	Dissolution / wt%	Charge zetapot / mV
TiO ₂ NM105	3.8	110	0	-23	50	6	0.15	-16	3.8	8	0	nd	33	0	38
BaSO ₄ NM220	1.8	87	0.12	-33	7.2	37	0.07	-4	2.7	45	0.10	nd	0	1.02	17
SiO ₂ _prec.	nd	82	1.18	-22	35	0	1.39	-1	3.4	36	0.55	nd	90	0.13	4

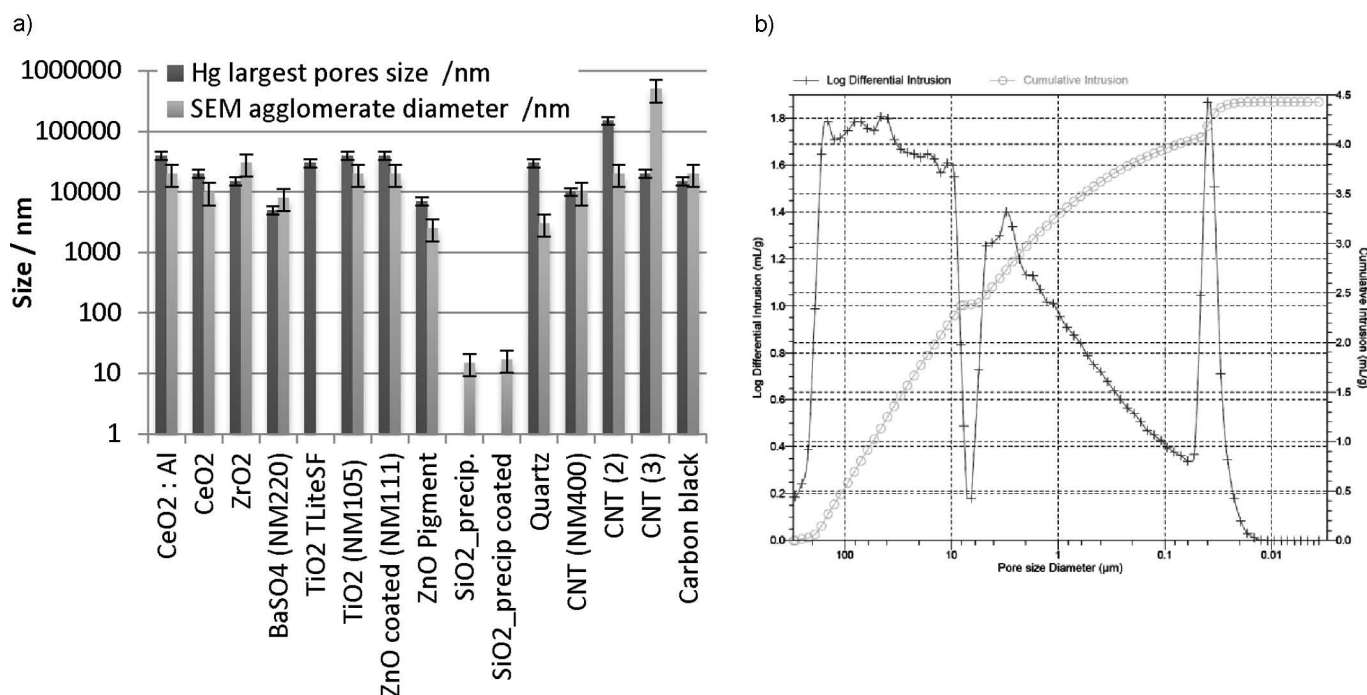


Fig. 2: Agglomerate size. a) Comparison of SEM and Hg porosimetry for all materials tested. b) Pore size distribution of TiO₂ NM105.

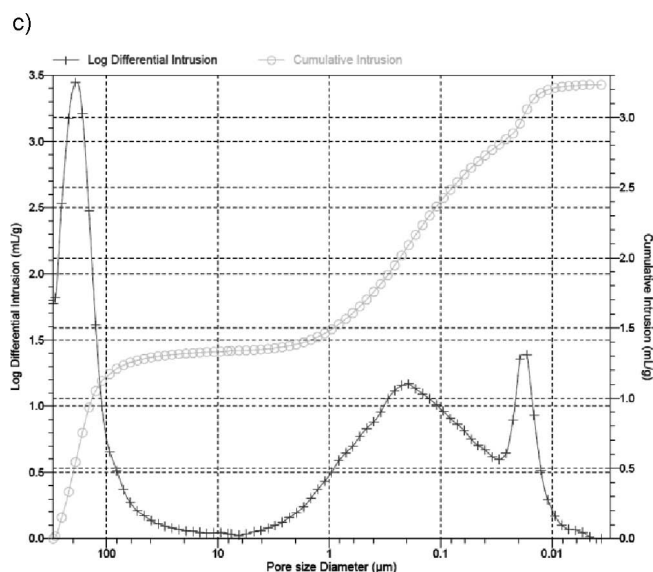


Fig. 2c: Agglomerate size. c) Pore size distribution of CNT (3).

Still in water (last row of Table 1), less than 0.1 ppm released Ti²⁺ ions are detected by means of ICP-MS in the supernatant of a 10 mg/ml suspension, stirred for 24 h and centrifuged to remove particulate material. However, in relevant media to simulate physiological conditions (Fig. 4), it can occur that TiO₂ NM105 recrystallizes: Especially in PSF to simulate the lysosome of macrophages a measurable mass of 0.15 wt% of the suspended material is detected as Ti ions, and although the nanomaterial clearly persists as such, the sphericity increases and larger primary particles emerge (Fig. 4). There is less dispersed fraction with diameters below 1 μm after incubation in PSF for 28 d than after incubation in PBS for 28 d or 0.1n HCl for 1 d.

For the other 14 materials, analogous descriptions of the physical-chemical properties can be read from Table 1. Their comparison along rows identifies outstanding properties.

a) Discussion 1: Comparison of materials

The two reference materials have the largest primary particles that clearly exceed 100 nm in diameter for ZnO pigment and for Quartz DQ12.

Several of the tested materials deviate strongly from globular nanoparticle shape: The CNTs are obviously nanotubes, but also TiO₂ T-Lite SF is a nanorod with an aspect ratio just above 3. ZnO-coated NM111 is polydisperse in shape, as confirmed (including all other structural results) by the OECD report of the PROSPECT project¹⁹. The two SiO₂ suspensions are closer to spheres than any other tested material, whereas Quartz has platelet shape.

All powder materials are observed in many-micron agglomerates with SEM and Hg porosimetry, but only BaSO₄ NM220 and CNT (3) form agglomerates with well-defined boundaries, which are spherical for BaSO₄ NM220 and pellets for CNT (3).

The crystallite sizes (XRD) are below 50 nm except for both ZnO variants and the Quartz DQ12, confirming again their significantly larger size. All ceramic materials are crystalline, except for the two amorphous suspended SiO₂ variants.

Many materials have several peaks in the Hg pore size distribution, pointing to a hierarchical assembly of primary particles into larger structures. This is e.g. the case for CNT (2) and CNT (3) (Fig. 2c), where the smallest pore peak at 20 nm confirms the outer diameter of an individual CNT as seen in TEM, but the second-smallest peak at 200 nm represents fibrilles or bundles, which then assemble further into large bundles up to 100 μm. Such an inter-

mediate structure is absent in the pore size distribution of CNT NM400. The same holds for TiO₂ NM105, where the smallest pore peak at 40 nm reflects the primary particles (Fig. 2b), with an unstructured distribution of pore sizes up to 100 μ m, confirming the loose fractal morphology of pyrolytic agglomerates.

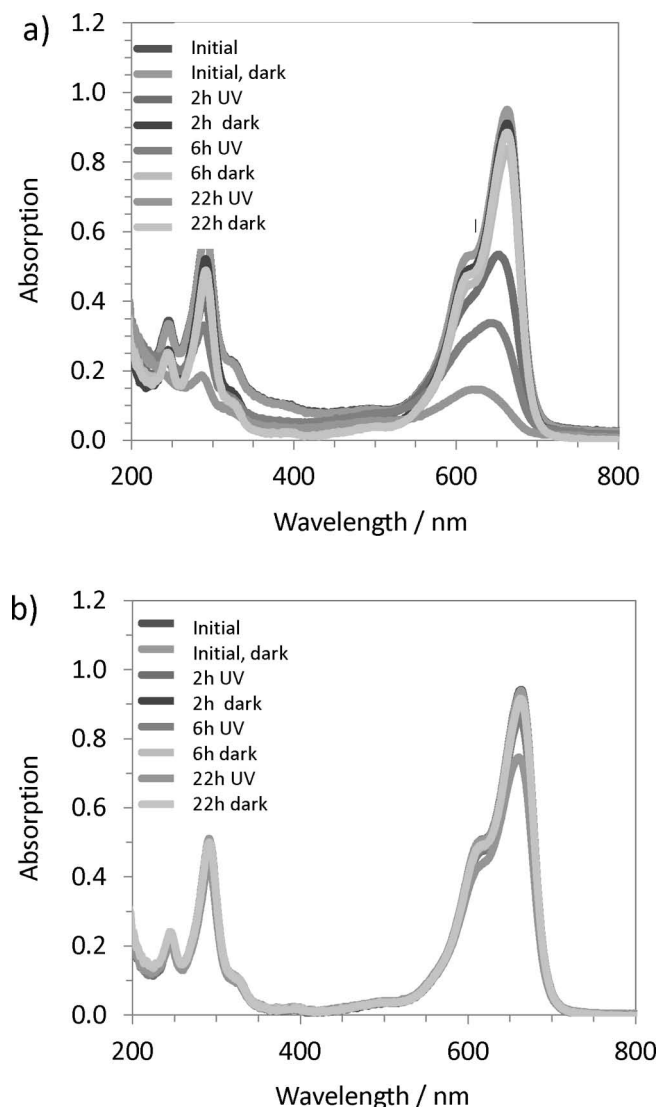


Fig. 3: Photocatalytic activity. Methylene Blue assay results a) for TiO₂ NM105 and b) for TiO₂ T-Lite SF.

The surface of all ceramic materials is significantly covered with organic components adsorbed from the surrounding atmosphere. These are unintended contaminations in most cases, except for the specific organic functionalizations on BaSO₄ NM220, TiO₂ T-Lite SF, ZnO-coated NM111 and SiO₂ precip. coated. The carbon-based materials are by far the purest of the materials tested with up to 99 % C. However, soot may be present on the CNTs, and would have to be regarded as an impurity. Only the CNT materials and the TiO₂ T-Lite SF have specific surface areas that exceed 100 m²/g.

Remarkable photocatalytic activity is observed for TiO₂ NM105, Quartz DQ 12 and ZnO pigments. The AlOOH coating of the TiO₂ surface that is applied in the T-Lite SF material is clearly effective in passivating the photocatalytic activity with a factor 5 lower photon efficiency (Fig. 3b). The excessive photon efficiency of our ZnO pigment is not

supported by different assays on closely related materials¹⁹. In contrast, the photon efficiency is vanishing for both SiO₂ precip. variants.

Strongly negative zeta potentials, confirmed by IEPs below pH 4, correlate with AANs close to unity in water (near-perfect dispersion), which then tend to agglomerate rather more than less in the presence of serum (DMEM/FCS): Such materials are BaSO₄ NM220, SiO₂ precip., SiO₂ precip. coated and Quartz DQ12. However, the SiO₂ precip. coated is outstanding with its perfect dispersion under both circumstances due to its electro-steric stabilization by the polymer corona that prevents the spontaneous adsorption of a protein corona. For the majority of ceramic powder materials, the AAN reduces in DMEM/FCS as compared to pure water. This effect can be safely attributed to their spontaneous adsorption of a protein corona that imparts at least partial stabilization by steric stabilization^{36,31,10}. Albumin is an especially effective dispersant for CNTs³⁷, but the limited concentrations of BSA in DMEM/FCS achieves a full individualization only for Carbon Black, and to a limited extent for CNTs.

As described above, the solubility of TiO₂ NM105 is below the detection limits in all media except PSF (simulating the lysosome of macrophages). However, the positive control SiO₂ precip. dissolves as expected (Fig. 4), including the least aggressive medium of PBS (simulating lung deposition). The solubility of BaSO₄ NM212 in all media, including PSF, is vanishing against the positive control, and noticeable only in 0.1n HCl. All materials, incl. TiO₂ NM105, undergo morphological changes by Ostwald ripening and recrystallization in PSF and HCl, but retain their crystallinity. In PSF, all tested materials tend to gel, but especially SiO₂ precip. loses its dispersed fraction while retaining its nanostructure. The lipids in FaS-SIF tend to agglomerate the nanomaterials. The BaSO₄ NM220 is remarkable in its high dispersed fine fraction, which may enhance the chance of translocation between body compartments.

b) Discussion 2: Methodical correlations and added value from disagreements

Some endpoints are determined in multiple redundancies: In Fig. 1 we plotted the results of all methods that provide a measure of the primary structures. Within the statistical error limits, the five methods of TEM-size, XRD-linewidth, Hg-peak, BET-surface, Hg-surface agree excellently for all ceramic and carbon-based materials. The exceptions include both the nano-ZnO (NM111) and the ZnO pigment, for which Hg intrusion finds a peak of implausibly small 5-nm pores. This artefact, possibly due to wetting of the sample, leads to a significant overestimation of the specific surface area from Hg as compared to BET. In general, however, Hg-intrusion can replace a BET measurement because it additionally provides a statistically relevant distribution of both primary particle sizes and of agglomerate sizes. The first reduces the necessity of counting hundreds of particles in TEM and the later essentially replaces SEM where statistics of agglomerates are very challenging. The agreement between the Hg largest pore size and SEM agglomerate evaluations is good enough to

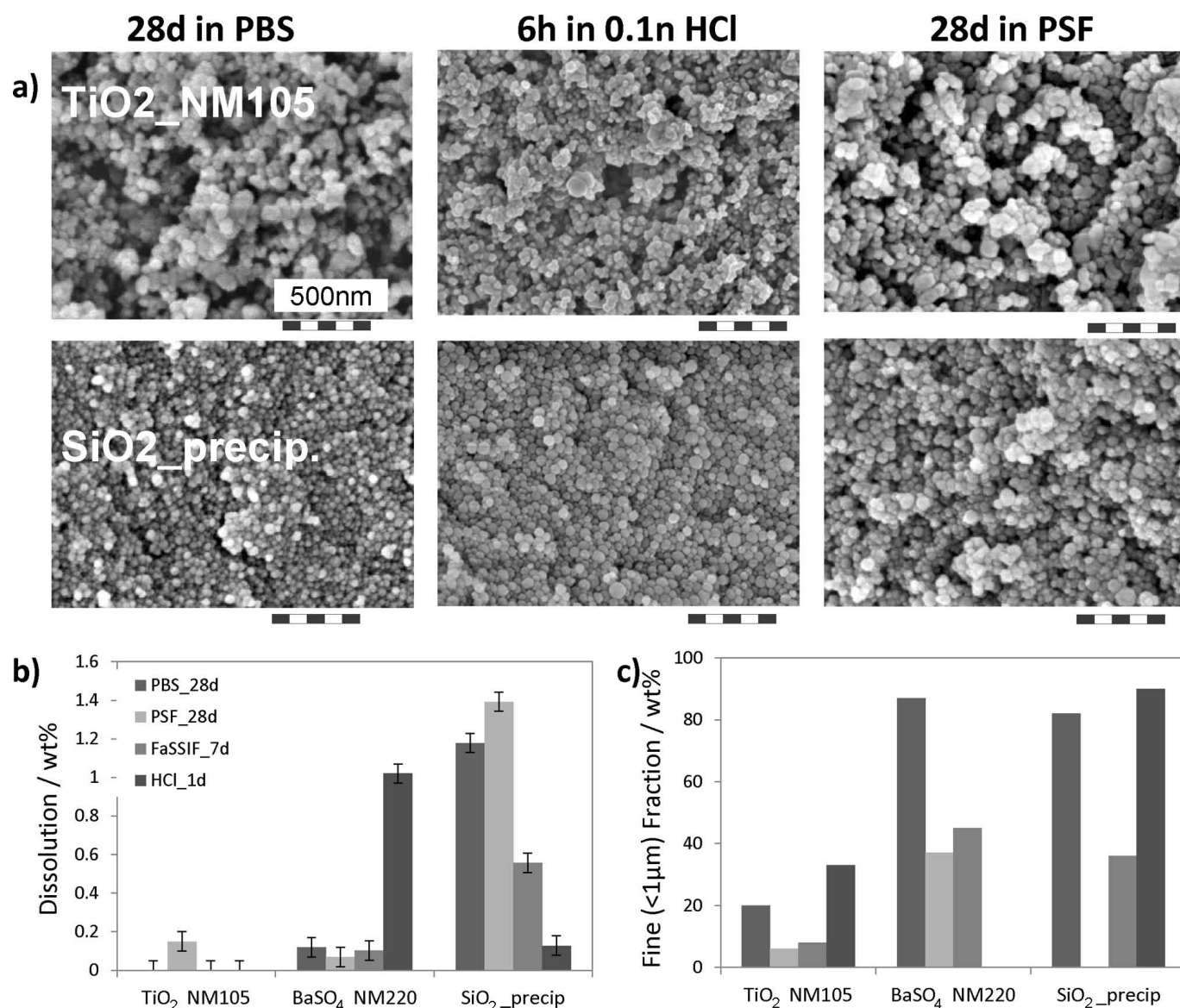


Fig. 4: Degradation *in situ*. a) SEM images of TiO₂ NM105 and SiO₂ precip. after incubation in the media indicated above each column. b) Relative dissolution as measured by ICP-MS on supernatants, for three selected materials. c) Remaining fine fraction of dispersed material as measured by AUC, supported by LD (see Table 2).

know the agglomerate dimensions as compared to primary particles (Fig. 2a).

For the two ZnO materials and for Quartz, also the XRD crystallites are significantly smaller than the sizes derived from TEM or BET, because the particles deviate strongly from sphericity. The observation of the XRD-linewidth disagreement may be regarded as an added value since it highlights the non-standard morphology. However, in all three cases that morphology was safely detected by means of TEM. The other endpoint extracted from the XRD measurement, the crystalline phase, may serve as proxy for the surface reactivity, but other endpoints such as the photocatalytic activity are easier to implement and closer to the actual mode of action. The necessity to measure XRD for regulatory purposes is hence lower than for the other endpoints.

To understand the material's fate and effects it seems to be essential to know its surface properties. There is indeed a good correlation between the surface charge and the *in situ* dispersability, and it is reasonable to assume that this

will have implications for the biokinetics, as observed e.g. for the SiO₂ precip. coated^{8, 17} and for CeO₂ variants³⁸. To achieve this good correlation it was essential to determine the *in situ* size distribution with an agglomerate-tolerant technique, hence not DLS (Dynamic Light Scattering)²⁵. Among the fractionating techniques we preferred AUC¹⁵ (see methods) over FFF (Field-Flow-Fractionation) because industrially relevant materials nearly always exceed the size dynamic range of FFF³⁹.

XPS analysis on as-received samples even with the C(1s) line shape analysis (Table 1) is not suitable to predict physiological interactions, owing to the high amount of loosely bound organic contaminations that hide the functional groups of the actual material surface or the organic functionalizations that presumably play a more important role for physiological or environmental interactions. This issue is even more pronounced for SIMS analysis where the information depth is confined to the topmost layer of the particle surfaces. However, the academic alternative of analyzing the *in-situ* spontaneously adsorbed corona is far

from standardization and is not mechanistically linked to biokinetics and physiological effects. In order to maintain all options for surface grouping approaches, XPS is still the best method if compared to the alternatives of SIMS (non-quantitative) or FT-IR (not surface-sensitive) or chemisorption (limited generality).

Some methods need to be further enhanced: The photocatalytic activity has value as an indicator of reactive materials but our current protocol cannot be applied to very hydrophobic materials. Assays in dry state are available and need to be validated for nanomaterials. The OECD methods for solubility are not generally applicable to nanomaterials³¹. We employed a protocol that relies on a hard centrifugation to remove particles before the ion content is determined. At 300 000 g the quantitative removal of nanoparticles is possible down to 2 nm diameter, but is time-consuming and needs to be verified. Attempts to identify impurities in the same supernatants by means of SIMS failed owing to either unspecific or omnipresent signals.

The standard biotic degradation tests employ the test material as energy source and are clearly of little use for ceramics and CNTs. As recognized by the OECD, any degradation phenomena need to be addressed by directly measuring degradation of the particles. In the present contribution, we explored a possible protocol based on incubation as suspension followed by elemental, colloidal and structural re-characterization. Positive (SiO₂) and negative (TiO₂) control materials dissolved (or not) as expected, but clearly on the morphological changes and their implications for fate and biokinetics much more validation is required.

Some OECD endpoints were not addressed here at all, because suitable methods were not accessible: Redox potential, Radical formation potential. Dustiness and Pour density were skipped owing to the large amounts of sample required.

Further reference must be given to the German "REACH-CLP helpdesk" operated by BAuA (Bundesanstalt für Arbeitsschutz und Arbeitsmedizin, Germany's Federal Institute for Occupational Safety and Health), which very recently released a further consolidation of the ECHA drafted endpoints, motivated by the need for established methods and the optimal use of existing data from e.g. quality assurance⁴⁰. We addressed all endpoints recommended by BAuA, and additionally we explored the surface reactivity (photocatalysis), porosity, and the *in situ* changes of persistence/solubility and dispersability. The methods that we chose are compliant with the BAuA methodical recommendations.

With the criteria of a) most endpoints from fewest measurements, as long as these remain consistent with complementary methods, b) successful differentiation between different materials, c) wide applicability, we prioritize the following set of endpoints and methods:

- Shape, size, aspect ratio of primary particles (TEM)
- Agglomeration = size distribution – dry, specific surface (Hg porosimetry, if not available to be replaced by BET + SEM)
- Surface charge (IEP with zeta-potential)
- Surface reactivity (photocatalytic activity)
- Dispersability (AUC or FFF in water and *in situ*)
- Dissolution *in situ* (ICP-MS of supernatants)

We recognize that further methods can be important, e.g. the detection of functionalizations and/or contaminations by means of XPS, but do not always differentiate between materials. Others are highly relevant for a full risk assessment (e.g. dustiness) or are expected to predict effects (e.g. radical formation potential) or to predict fate (e.g. degradation), but for these properties efficient methods still need to be validated and commercialized.

V. Conclusions

Starting from methods recommended in the REACH nanospecific guidance draft R7.1, we performed a comparative characterization on 20 physical-chemical endpoints of 15 materials, including some from the OECD sponsorship testing program. We proposed criteria to prioritize a set of critical endpoints and suitable methods, and found the result to be compliant with the practical guidelines of German authorities⁴⁰, to which our assessment suggests slight modifications. As a very first correlation between properties and effects that were observed in the NanoCare and nanoGEM projects, we note that those materials which stood out with increased mobility in the body and exceptionally low or high inflammation potency actually differentiate also by the physical-chemical endpoints prioritized here: reactive materials (Quartz, TiO₂, ZnO), materials that release ions and are only partially persistent *in situ* (ZnO, SiO₂ precip.), fiber materials (certain kinds of CNTs), organic functionalizations (BaSO₄ NM220, SiO₂ precip. coated). The present contribution only provides the basis for a more detailed investigation of such correlations on the way towards grouping approaches and nano-QSARs.

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