The impact of synthetic amorphous silica (E 551) on differentiated

Caco-2 cells, a model for the human intestinal epithelium

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¹ ADI: acceptable daily intake; ASTM: American society for testing and materials; AU: airy unit; BSA: bovine serum albumin; CAS No: chemical abstracts service registry number; CdSO₄: cadmium sulphate; CLSM: confocal laser scanning microscopy; CO₂: carbon dioxide; DAPI: 4',6-diamidino-2-phenylindole; DCF: dihydrodichlorofluorescein diacetate; ddH₂O: double distilled water; DG: distorted grid; DLS: dynamic light scattering; DSMZ: Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures; DSS: dextran sulphate sodium; E 551: food additive silicon dioxide; ECD: equivalent circular diameter; EDTA: ethylenediaminetetraacetic acid; EDX: energy dispersive X-ray spectroscopy; EFSA: European Food Safety Authority; EU: endotoxin unit; FCS: fetal calf serum; H₂DCF-DA: 2',7'-dichlorodihydrofluorescein diacetate; HBSS: Hank's balanced salt solution; HMDS: hexamethyldisilazane; HTS: high throughput screening; ISO: International Organization for Standardization; M-cells: Microfold cells; MEM: minimum essential medium; MWCNT: multi-walled carbon nanotubes; P_{app}: apparent permeability coefficient; PBS: phosphate buffered saline; PET: polyethylene terephthalate; PS-amine: amine modified polystyrene nanoparticles; ROS: reactive oxygen species; RT: room temperature; SAS: synthetic amorphous silica; SEM: scanning electron microscopy; Sin-1: 3-morpholinocydonimine hydrochloride; SiO₂: silicon dioxide; TEER: transepithelial electrical resistance; TEM: transmission electron microscopy; ToF-SIMS: time-of-flight secondary ion mass spectrometry; VCM: volumetric centrifugation method; ZO-1: zonula occludens-1

Abstract

For several decades, food-grade synthetic amorphous silica (SAS) have been used as a technological additive to reduce caking of food powders. Human exposure is thus inevitable and safety concerns are taken seriously. The toxicity of silica in general and SAS in particular has been studied extensively. Overall, there is little evidence that food-grade SAS pose any health risks to humans. However, from the available data it was often not clear which type of silica was used. Accordingly, the latest report of the European food safety authority requested additional toxicity data for well-characterised "real food-grade SAS".

To close this gap, we screened a panel of ten well-defined, food-grade SAS for potential adverse effects on differentiated Caco-2 cells. Precipitated and fumed SAS with low, intermediate and high specific surface area were included to determine structure-activity relationships.

In a physiological dose-range up to 50 µg/ml and 48 hours of incubation, none of the materials induced adverse effects on differentiated Caco-2 cells. This held true for endpoints of acute cytotoxicity as well as epithelial specific measures of barrier integrity. These results showed that despite considerable differences in production routes and material characteristics, food-relevant SAS did not elicit acute toxicity responses in intestinal epithelial cells. ~~strongly indicate that independent of the production process and the specific surface area, SAS materials do not induce acute toxicity after oral exposure.~~

Key Words:

in vitro toxicology, food-grade synthetic amorphous silica (E 551), differentiated Caco-2 cells, dosimetry

49 1 Introduction

50 Synthetic amorphous silica (SAS) is described as a white, fluffy powder or granules
51 with the chemical formula SiO_2 (silicon dioxide; silica) (EFSA, 2018). Three main types
52 of silica exist: crystalline silica, amorphous silica and SAS (Fruijtier-Pölloth, 2012). SAS
53 are registered under the CAS No 7631-86-9 and are further divided in silica gel,
54 precipitated, pyrogenic and colloidal silica. Three main types of silica exist under the
55 same CAS No (7631-86-9): crystalline silica, amorphous silica and SAS (Fruijtier-
56 Pölloth, 2012). SAS are further divided in silica gel, precipitated, pyrogenic and
57 colloidal silica. Among those only the gel, precipitated and pyrogenic forms but not the
58 colloidal form, are authorised as food additives under the European Union registration
59 number E 551 (European Commission, 2008a). E 551 is widely used as a
60 technological additive due to its anti-caking properties (Fruijtier-Pölloth, 2016; Kurfess
61 et al., 2005; Müller et al., 2008; Ruppel et al., 2009).

62 *In vivo* studies in rodents have shown that oral exposure to food-grade SAS (E 551)
63 did not induce systemic or local toxicity (Fruijtier-Pölloth, 2016). However, of the many
64 existing toxicity studies (*in vitro* as well as *in vivo*) only few applied real food-grade
65 SAS and specified what type of silica has been used (for a comprehensive review see
66 Fruijtier-Pölloth, 2016; Sohal et al., 2018). Hence, the EFSA panel on food additives
67 and nutrition sources concluded in its 2018 report on "The re-evaluation of silicon
68 dioxide as a food additive" that there is a need for an improved particle characterisation
69 of E 551. Furthermore, they requested additional toxicity data on real E 551 in its
70 various available and authorised forms. According to the panel report, the availability
71 of biological and toxicological data on E 551 is still limited. Moreover, the use of

different SAS forms with distinct physico-chemical properties further prevented a comprehensive and systematic toxicological evaluation. Therefore, the current acceptable daily intake (ADI) 'not specified' could still not be confirmed.

In order to take this issue forward, appropriate model systems are needed that reliably detect potential adverse effects and allow for predicting toxicity in humans. The utilization of *in vitro* cell culture models to replace or at least complement *in vivo* animal toxicity testing is the integral part of the United States National Research Council report "Toxicity Testing in the 21st Century: A Vision and a Strategy" (National Research Council, 2007). This report clearly promotes a paradigm shift in toxicology from classical, phenomenological oriented *in vivo* animal testing towards the implementation of *in vitro* methods to understand mechanisms and molecular pathways underlying the observed adverse effects. Likewise, in Europe REACH (European regulation concerning the registration, evaluation, authorisation and restriction of chemicals) encourages companies to use alternative approaches before deciding on any testing on vertebrate animals (European Commission, 2006). For that purpose, the European Commission operates the European centre for the validation of alternative methods (ECVAM), which is a global leader in the field. Ideally, such an initial *in vitro* toxicity screening approach comprises both *in vivo* relevance (=predictive power) as well as high-throughput capabilities.

A commonly used *in vitro* model for the human intestinal tract is the human colorectal adenocarcinoma cell line Caco-2. Upon reaching confluence on a microporous membrane, these cells have been shown to express characteristics of enterocytic differentiation, such as polarization and the presence of brush boarder microvilli and tight junctions (Jumarie and Malo, 1991; Lea, 2015; Pereira et al., 2016). These features render them physiologically more relevant compared to the undifferentiated

97 state (Pietroiusti et al., 2017). Furthermore, undifferentiated Caco-2 cells are generally
98 more sensitive towards cytotoxic injuries (Gerloff et al., 2013; Pietroiusti et al., 2017;
99 Song et al., 2015), which could lead to an overestimation of the hazard of the
100 respective compounds. Additionally, differences between differentiated and
101 undifferentiated Caco-2 cells regarding their uptake behaviour of nanomaterials have
102 been reported. While undifferentiated cells internalized graphene sheets (Kucki et al.,
103 2017), quantum dots (Peuschel et al., 2016) as well as cerium oxide nanoparticles (Vila
104 et al., 2018b) their differentiated counterparts did not. This is in line with observations
105 *in vivo* that enterocytes preferentially ingest soluble but not particulate factors (as for
106 example soluble protein antigens versus particulate or aggregated antigens (Shao et
107 al., 2001)). This therefore demonstrates that differentiated Caco-2 cells better reflect
108 the *in vivo* functionality of enterocytes (Lea, 2015; Shao et al., 2001; Song et al., 2015).

109 Besides the choice of a suitable model system, determining the relevant dose ranges
110 and furthermore matching *in vitro* with *in vivo* dosing pose additional challenges in
111 toxicology. Real-life human exposure scenarios have to be considered, that allow for
112 a worst-case experimental dosing regime. Based on the amount of silica in different
113 food categories (e.g. Dekkers et al., 2011) as well as different consumption patterns of
114 the population the daily SiO₂ intake of an "average 70 kg adult" can vary tremendously
115 from 35 mg (Lomer et al., 2004) up to 3500 mg (EFSA, 2018). Similarly, different
116 publications on the total human gut mucosal surface area report values ranging from
117 32 m² (Helander and Fändriks, 2014) to 200 m² (DeSesso and Jacobson, 2001).
118 Assuming 100% of the SiO₂ intake to deposit on the gut surface a maximum
119 concentration of 11 µg/cm² per person per day (3500 mg/32 m²) as well as a minimum
120 concentration of 0.02 µg/cm² per person per day (35 mg/200 m²) can be calculated.
121 This gives a first rough estimate of a physiologically relevant dosage range for *in vitro*

studies when considering a single exposure scenario and acute toxicity measurements.

The present study aimed to close the knowledge gap of comparative *in vitro* toxicity data for different types of food-grade SAS materials, authorized and applied as food additive E 551. Differentiated Caco-2 mono-cultures were chosen as a pre-screening model to specifically address the impact of SAS on enterocytes, which represent the most abundant epithelial cell type of the intestine and exert many crucial functions. In order to resolve potential structure-activity relationships, we used a panel of 10 food-grade SAS materials covering the two most prominent production processes, wet and thermal. In addition, both groups (precipitated and fumed silica, respectively) contained materials with low, middle and high specific surface area. According to the ROS-paradigm (Roesslein, Hirsch, Kaiser, Krug, & Wick, 2013 and references therein) we analysed the production of ROS as a first potential stressor of SAS-cell interaction and cell viability/cell death as the last and most deleterious effect. Barrier integrity served as an epithelial specific endpoint. Physiological dose-ranges were verified applying the distorted grid (DG) model (DeLoid et al., 2017) as well as time-of-flight secondary ion mass spectrometry (ToF-SIMS). All assays were well established for the use with nanomaterials and control experiments were implemented to exclude unintended interference reactions of nanomaterials with the assay components (Belyanskaya et al., 2007; Casey et al., 2007; Hirsch et al., 2011; Kroll et al., 2012; Monteiro-Riviere et al., 2009; Vietti et al., 2013). All SAS materials were comprehensively characterised in line with the relevant EFSA guidance document (Hardy et al., 2018).

2 Materials and Methods

2.1 Particle synthesis

Ten food-grade SAS materials were kindly provided by Evonik Resource Efficiency GmbH (Hanau, Germany). All SIPERNAT® ~~types are materials (=~~ precipitated silica ~~and)~~ were produced by wet process, while all AEROSIL® ~~types are materials (=~~ fumed silica ~~and)~~ were produced by flame hydrolysis (EFSA, 2018; IPTS/EC, 2007). The materials were applied without further acid pre-treatment, as it has been shown that acid pre-treatment did not alter the characteristics of the SAS materials (Maier et al., 2013).

2.2 Particle dispersion

10 mg/ml SAS stock dispersions were prepared in double distilled water (ddH₂O), vortexed and dispersed using an ULTRA-TURRAX® T25 (IKA, Staufen, Germany) at 14600 rpm for 1 min. Stock dispersions were stored at room temperature (RT) for up to 12 weeks. ULTRA-TURRAX® treatment of stock dispersions was repeated prior to any SAS treatment. The dispersion with ULTRA-TURRAX® T25 results in a particle size distribution found in food matrix (Contado et al., 2013; Maier et al., 2015 as cited in EFSA et al., 2018).

2.3 Particle characterisation

Particle characteristics are summarised in table 1. Further details can be found in (Retamal Marín et al., 2018).

The specific surface area of SAS powders was determined by Brunauer, Emmet and Teller (BET) method according to ISO 9277 based on the absorption of gaseous nitrogen (Brunauer et al., 1938; ISO: Geneva, 2010).

Particle size distributions of aqueous dispersions (retrieved from ULTRA-TURRAX® treatment) diluted in ddH₂O to 50 µg/ml were measured by dynamic light scattering (DLS) at room temperature in disposable cuvettes (UV-transparent, Sarstedt AG+Co, Germany) using a Zetasizer, Malvern Nano-ZS90 (Malvern Instruments Ltd., Worcestershire, United Kingdom). Z-average values given in table 1 correspond to the mean and standard deviation of two independent measurements with three technical replicates each. Determination of size distribution in medium was not possible since the suspensions were polydisperse and hence, sedimentation of larger agglomerates would results in an underestimation of particle sizes.

Agglomerate effective densities were measured in 0.1 mg/ml dispersions in ultrapure water by volumetric centrifugation method (VCM) as described before (DeLoid et al., 2014).

Point of zero charge was determined in dispersions according to ISO 787-9 (ISO: Genva, 1981). Zeta potential and point of zero charge were measured with an electroacoustic sensor at a solid density of 2.1 g/ml using a DT-102 instrument (Dispersion Technology, New York, USA) (Retamal Marín et al., 2017). Experiments in cell culture medium were not performed due to high ionic strength and conductivity, which can considerably interfere with the measurements.

The density of the SAS materials was determined according to OECD test guideline no. 109 (OECD, 1995) and European Union test method A. 3 Council Regulation (EC) no. 440/2008 of 30 May 2008 by gas displacement pycnometry (Accupyc 1330, Micromeritics) (European Commission, 2008b). For the calculation of density, the mass of the SAS materials was measured with an analytical balance. The method is described in DIN 66137-2 (Deutsches Institut für Normung, n.d.) and Ph.Eur.2.9.23 (Council of Europe, 2014).

Sizes of primary structures as well as aggregates were measured with a Jeol 2010F transmission electron microscope at 200 keV acceleration voltage and a F416 camera (TVIPS, Gauting, Germany) with ITEM software (EMSIS GmbH, Münster, Germany). Therefore, 2000 and 1000 single elements were analysed, respectively. After energy input by liquid phase dispersion and agitation by ultra sonication, a standard transmission electron microscopy (TEM) sample preparation procedure for powders was applied related to ASTM (American Society for Testing and Materials) D 3849–07.

2.4 Endotoxin measurement

Endotoxin contaminations in SAS dispersions in ddH₂O were detected with the PYROGENT™ Plus Gel Clot LAL Assays (sensitivity 0.03 EU/ml) according to the manufacturer's instructions (Lonza). Serial dilutions from 125 µg/ml to 15.6 µg/ml were assessed as described before (Kucki et al., 2017).

2.5 Cell culture

The human colorectal adenocarcinoma cell line Caco-2 was obtained from the German collection of microorganisms and cell cultures (DSMZ, Braunschweig, Germany). Cells were maintained in minimum essential medium (MEM) (Sigma), supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 1% (v/v) penicillin, streptomycin, neomycin and 1% (v/v) non-essential amino acids (all from Sigma) (hereafter called "complete cell culture medium") at 37°C and 5% CO₂ and 95% humidity, hereafter called "standard growth conditions". Caco-2 cells were grown in 75 cm² cell culture flasks (TPP) until reaching 80% confluence and subcultured using trypsin-EDTA (Sigma).

2.6 Caco-2 cell differentiation

To obtain a differentiated Caco-2 monolayer, cells were seeded at a concentration of 2×10^4 cells/mm² into the apical compartment of microporous polyester (PET) membrane inserts (1 μ m pore; 12-well inserts: ThinCert™ (Greiner); 96-well high throughput screening (HTS) inserts: Corning® HTS Transwell®-96, (Corning)). The basolateral compartment was filled with complete cell culture medium and cells were allowed to differentiate under standard growth conditions for 21 days. Medium was changed every other day. Unless stated otherwise, all experiments were conducted on differentiated Caco-2 cells.

2.7 Treatment of differentiated Caco-2 cells

Treatment volumes in the apical compartments of 12- and 96-well inserts were calculated to match computed medium heights of 5.2 mm in both well sizes to achieve identical dosing conditions. Therefore, the 12-well inserts received 590 μ l SAS suspensions/well apical volume and the HTS 96-well inserts received 75 μ l/well apical volume. Cells were treated in complete cell culture medium made from phenol red free MEM (Gibco).

2.8 ROS formation (DCF assay)

Intracellular ROS were detected using 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA, Molecular Probes) as reported previously with minor changes (Bachmatiuk et al., 2013; May et al., 2018; Roesslein et al., 2013). Cells were differentiated in HTS 96-well inserts as described above. Prior to treatment, cells were loaded with 50 μ M H₂DCF-DA in Hank's balanced salt solution (HBSS) for 60 min at standard growth conditions. After two washing steps with pre-warmed HBSS, cells were treated with the following concentrations of SAS in HBSS 0, 3.125, 6.25, 12.5, 25

and 50 µg/ml. The 0 µg/ml samples received an equivalent volume of ddH₂O in HBSS as a solvent control. Sin-1 (3-morpholinosydnonimine hydrochloride, Sigma) at a concentration of 50 µM served as the chemical positive control. Multi-walled carbon nanotubes (MWCNT; Bayer Technologies Service) have been shown to induce intracellular ROS formation (Ghitescu et al., 2018) and thus served as the particle positive control at a concentration of 20 µg/ml in this study. Material properties have been extensively characterized before (Thurnherr et al., 2009). Fluorescence was measured in a multi-well plate reader (Mithras², Berthold Technologies) after one, two, three and four hours at 485/528 nm (excitation/emission). Cells were maintained under standard growth conditions between measurements. Only values from the 2 h time point are presented in the results and supplements sections. Data shown represent the mean and standard deviations of three independent experiments with four technical replicates each.

2.9 Cell viability (resazurin assay)

The culture medium was removed from differentiated Caco-2 cells in HTS 96-well inserts and cells were treated with concentrations of 0, 3.125, 6.25, 12.5, 25 and 50 µg/ml SAS for 24 and 48 h in 75 µl fresh complete phenol red free cell culture medium. Cadmium sulphate (CdSO₄, Sigma) was used in concentrations of 0, 1, 10, 100, 1000, and 10000 µM and served as a chemical positive control. Amine modified polystyrene nanoparticles (PS-amine) (Bangs Laboratories, PA02N, Indiana, USA) have been characterized before and reduced cell viability *in vitro* dose-dependently (Elliott et al., 2017). Therefore, these particles served as the particle positive control at concentrations of 0, 12.5, 25, 50, 100, and 200 µg/ml in this study. All 0 µg/ml samples received an equivalent volume of ddH₂O in medium as solvent control. The resazurin stock solution (1 mg/ml in ddH₂O) was prepared freshly for every experiment. 24 and

48 h post-exposure 10 µL of a 21.27 µg/ml pre-dilution resazurin stock in phosphate buffered saline (PBS) were added directly to each well resulting in a final concentration of 2.5 µg/ml resazurin in complete phenol red free cell culture medium. The cells were incubated for 2 h at standard growth conditions and the fluorescence was measured with a multi-well plate reader at 530/585 nm (excitation/emission). Data shown represent the mean and corresponding standard deviations of three independent experiments with four technical replicates each.

2.10 Evaluation of the barrier integrity

2.10.1 Measurement of transepithelial electrical resistance (TEER)

The differentiation process of ~~the all~~ Caco-2 monolayers cultures grown in 12-well inserts was controlled by TEER measurements after 7, 14 and 21 days and at the end of the experiment using an Epithelia Voltohmmeter (EVOM) with sterilised STX2 electrodes (World precision, Instruments, Sarasota Florida, USA). Only Caco-2 cell monolayers with TEER values higher than 700 Ωcm² on day 21 were used for experiments. To better depict the TEER development of the cultures over time, additional values on days 3, 6, 10, 13, 16 and 19 were measured in one experiment with 12 replicates.

The impact of SAS, ddH₂O (solvent control) and PS-amine (particle positive control) on the barrier integrity of differentiated Caco-2 cells was studied by measuring the TEER after 48 h of treatment. The tight junction disruptor Dextran Sulphate Sodium (DSS; Sigma; 2% and 5%) (Gangwar et al., 2017) and the ROS inducer Luperox® (Sigma; 12.5, 25 and 50 µM; (Chamoto et al., 2017)) served as positive controls. TEER values were measured after 1 h of treatment with the respective control substances.

2.10.2 Translocation study

Differentiated Caco-2 cells in 12-well inserts were exposed to ddH₂O (solvent control), 200 µg/ml PS-amine or 50 µg/ml SAS for 48 h at standard growth conditions. Thereafter the medium was removed from both compartments and replaced apically with 1 ml of a 5 µM sodium fluorescein solution in fresh complete phenol red free medium. The basolateral compartment received 1.5 ml fresh complete phenol red free medium only. After 5 h under standard growth conditions, three 100 µl samples were collected from the apical as well as the basolateral side. The fluorescence was measured with a multi-well plate reader (Mithras², Berthold Technologies) at 490/520 nm (excitation/emission). The apparent permeability coefficient (P_{app}) was calculated using equation number 1 (Aengenheister et al., 2018; Kowapradit et al., 2010). Therein dQ/dt is the rate of sodium fluorescein appearance on the basolateral side, V the volume in the basolateral compartment, A the surface area of the monolayer and C_0 the initial sodium fluorescein concentration on the apical side.

$$P_{app} = \frac{dQ}{dt} \times \frac{V}{A \times C_0} \quad (1)$$

2.11 Immunostaining of tight junction proteins

Differentiated Caco-2 cells in 12-well inserts were exposed to 50 µg/ml SAS or ddH₂O (solvent control) for 48 h. TEER values were measured and the cells were washed twice with PBS and fixed with 4% paraformaldehyde for 20 min at RT. The cells were washed with PBS and incubated with 30% sucrose at 4 °C overnight. Thereafter, cells were permeabilized with 0.1% Triton X-100 for 15 min, washed three times with PBS and non-specific binding sites were blocked for 30 min with 5% BSA in PBS. Cells were incubated with Alexa Fluor® 488 conjugated mouse anti-ZO 1 antibody (Invitrogen 339188; 1:50) and rabbit anti-Occludin antibody (Invitrogen 40-4700; 1:100) in 1% BSA

in PBS at 4 °C overnight. After three washing steps in PBS, cells were incubated for 1 h with goat anti-rabbit Alexa Fluor® 555 (Invitrogen; A21428; 1:400) and phalloidin Alexa Fluor 633 (Invitrogen A22284; 1:50) in 1% BSA in PBS. After additional three washing steps in PBS, nuclei were counterstained with 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI, Sigma D9542) in PBS for 10 min at RT. Caco-2 monolayers were carefully mounted with Mowiol® 4-88 (Sigma 81381) and covered with a glass coverslips, which was sealed with nail polish. A confocal laser scanning microscope (Zeiss microscopes, Jena, Germany) with a C-Apochromat 40x/1.2 W Corr M27 objective was used to obtain z-stack images. The confocal pinhole was set to 1 AU to optimise the z-sectioning in the confocal mode. Images and z-stacks were recorded sequentially with a z-interval of 0.38 µm, resulting in a maximum of 30 images per z-stack.

2.12 Evaluation of the cellular adhesion of SAS

2.12.1 Dosimetry Simulations

The Matlab code for the distorted grid (DG) model was used as supplied by DeLoid and co-workers 2017 (DeLoid et al., 2017). Fixed model parameters are summarised in supplementary table S1. Particle size distributions were determined by DLS as described above using a Zetasizer, Malvern Nano-ZS90 (Malvern Instruments Ltd., Worcestershire, United Kingdom). Volume-weighted size distributions were used as input values for the DG model and are summarised in supplementary file 2. Agglomerate effective densities were measured using VCM (see 2.3) and are summarised in table 1. All simulations were run assuming a non-sticky bottom as recommended by DeLoid and co-workers (DeLoid et al., 2015).

2.12.2 Scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDX)

Differentiated Caco-2 cells in 12-well inserts were exposed to ddH₂O (solvent control), 50 µg/ml AEROSIL® 380 F or SIPERNAT® 22 S for 48 h. After exposure the cells were washed twice in pre-warmed PBS and fixed with modified Karnovsky fixation solution (4 g paraformaldehyde, 50 ml ddH₂O, 5 ml glutaraldehyde 50%, 45 ml PBS without glucose and pH 7.4) for 1 h at RT (for more information please refer to Kucki et al. 2017). Briefly, samples were washed in PBS and dehydrated by ascending ethanol series (50–100% ethanol) followed by 30 min treatment with hexamethyldisilazane (HMDS). The cells were dried overnight in a fume hood and transferred to SEM sample holders with conductive adhesive tapes on the next day. Samples were sputter-coated (Sputter Coater Leica EM ACE600) with gold-palladium (ratio Au/Pd: 80/20, 10 nm thickness). SEM analysis was performed with a Hitachi S-4800 SEM operating at 2 kV and EDX analysis was performed with an INCA X-Sight EDS (Oxford Instruments) detector operating at 20 kV.

2.12.3 ToF-SIMS

Differentiated Caco-2 cells in 12-well inserts were exposed to ddH₂O (solvent control), 50 µg/ml AEROSIL® 380 F or SIPERNAT® 22 S for 48 h. Thereafter, cells were fixed with 4% paraformaldehyde in ddH₂O for 1 h at RT and dehydrated by an ascending ethanol series (50 – 100% ethanol).

ToF-SIMS was performed on a "ToF-SIMS.5" instrument from ION-TOF GmbH. Measurements were performed with a Bismuth (Bi) primary ion beam (25 keV) and a target current of 0.35 pA (cycle time: 50 µs). Spectra were recorded in high current bunched (HCBU) mode using Bi₃⁺ on a 500 × 500 µm² area with a resolution of 128 × 128 px² with 50 scans. The spectra intensities were normalised to the peak of

CH₂N and averaged over 5 different positions on the sample for better statistics. Two independent samples were analysed.

2.13 Statistics

Results are presented as means $\pm$ standard deviations of three independent experiments, which were run with at least two technical replicates each. Statistical analysis was conducted with GraphPad Prism 6 software using a one-way analysis of variance (ANOVA; 95% confidence interval) followed by the Dunnett's or Bonferroni's multiple comparisons test.

3 Results

3.1 Material characterisation

Physicochemical properties of all 10 SAS materials are summarised in table 1. Food-grade SAS material production results in nanostructured aggregates, which are the smallest isolable and dispersible units of E 551 (Fruijtier-Pölloth, 2016) and do not show any internal phase borders (Albers et al., 2015). Weak interaction forces between aggregates lead to the formation of even bigger agglomerates during the production process. TEM allowed us to estimate the sizes of the primary structures, which are defined as the smallest visually identifiable structures within an aggregate. Diameters of the primary structures ranged from 3 nm (SIPERNAT® 50 and 50 S) to 41 nm (AEROSIL® OX50). Likewise, aggregate sizes were determined by TEM and the retrieved equivalent circular diameters ranged from 45 nm (SIPERNAT® 2200) to 276 nm (SIPERNAT® 350). Representative TEM micrographs of SIPERNAT® 22 S and AEROSIL® 380 F are exemplarily shown for each material class (precipitated and fumed, respectively) in supplementary figure S1. The hydrodynamic diameters of SAS

382 agglomerates in ultrapure water were determined by DLS and revealed z-average
383 values from 294 nm (AEROSIL® 200 Pharma) to 747 nm (SIPERNAT® 50 S) (table
384 1). Respective volume-weighted size distributions (supplementary file 2) were used for
385 dosimetry simulation *in silico*. Likewise, material densities as well as agglomerate
386 effective densities were used for dosimetry simulations. Material density values were
387 similar within the two material classes; for all SIPERNAT® materials ranging from 2.12
388 to 2.16 g/cm³ and for all AEROSIL® materials ranging from 2.29 to 2.38 g/cm³.
389 Agglomerate effective density values ranged from 1.07 g/cm³
390 (AEROSIL® 200 Pharma) to 1.65 g/cm³ (SIPERNAT® 22). The specific surface area
391 of the materials varied considerably between 45 m²/g and 460 m²/g. For both material
392 classes samples with low (~50 m²/g), middle (~200 m²/g) and high (~400 m²/g) specific
393 surface area were included in the study design to be able to correlate potential
394 biological effects to this toxicologically important material property (Oberdörster et al.,
395 2005; Schmid and Stoeger, 2016). The zeta potentials were measured with an
396 electroacoustic sensor for the precipitated SAS between -41.2 mV and -13.7 mV and
397 for the fumed SAS between -34.0 and -10.6 mV. Whereas the point of zero charge of
398 the SAS materials was measured around pH 2.0 with the minimum of pH 1.5 and
399 maximum of pH 2.4. All 10 SAS materials were negative for endotoxin up to 50 µg/ml
400 (data not shown).

Material	Code ^I	Production process	Primary structure size/ nm ^{II}	Aggregate size (ECD)/ nm ^{II}	Specific surface area/ (m ² /g) ^{III}	Material density/ (g/cm ³) ^{IV}	Agglomerate effective density/ (g/cm ³) ^V	Agglomerate size/ nm ^{VI}	Point of zero charge/ pH ^{VII}	Zeta potential/ mV ^{VIII}
SIPERNAT® 350		precipitated	30.3 ± 6.8	276.3	55	2.15	1.33	623 ± 15	1.7	n.a.
SIPERNAT® 22	P-5	precipitated	10 ± 2.6	82.2	180	2.12	1.65	325 ± 37	1.7	-37.1
SIPERNAT® 22 S		precipitated	10 ± 2.6	82.2	185	2.14	1.20	743 ± 38	2.0	<u>-37.1</u>
SIPERNAT® 2200		precipitated	9 ± 1.4	44.9	185	2.12	1.25	324 ± 13	1.5	n.a.
SIPERNAT® 160	P-1	precipitated	12.2 ± 2.7	58.3	180	2.16	1.18	555 ± 32	1.7	-41.2
SIPERNAT® 50	P-2	precipitated	3.1 ± 0.7	59.8	460	2.12	1.15	645 ± 79	1.7	-13.7
SIPERNAT® 50 S		precipitated	3.1 ± 0.7	59.8	460	2.12	1.16	747 ± 86	1.7	<u>-13.7</u>
AEROSIL® OX50	F-1	fumed	41.4 ± 18.3	233.7	45	2.32	1.25	299 ± 6	2.3	-34.0
AEROSIL® 200		fumed	7.8 ± 1.8	161.1	210	2.38	1.07	294 ± 5	2.3	-11.8
Pharma										

AEROSIL® 380 F	F-3	fumed	8 ± 2.7	101.9	390	2.29	1.09	318 ± 15	2.4	-10.6
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401 **Table 1:** Properties of food-grade SAS materials. ^IThe same materials were used in a recent study by Wiemann and co-workers
 402 (Wiemann et al., 2019) under the respective codes given here. ^{II}Analysed by TEM. The arithmetic mean and corresponding standard
 403 deviations from 2000 and 1000 items are given for primary structure and aggregate sizes, respectively. ECD: equivalent circular
 404 diameter. ^{III}Assessed by N₂-BET. ^{IV}Assessed by gas displacement pycnometry. ^VAssessed by volumetric centrifugation method from
 405 0.1 mg/ml suspensions in ddH₂O. ^{VI}Z-average values as assessed by DLS from 0.05 mg/ml suspension in ddH₂O. The mean and
 406 corresponding standard deviations from two independent measurements with three technical replicates each are given. ^{VII/VIII}Point of
 407 zero charge as well as zeta potential were measured with an electroacoustic sensor at a solid density of 2.1 g/ml.

3.2 Characterisation of the *in vitro* model

To assess the influence of SAS materials on the human gastro-intestinal tract differentiated Caco-2 cells were used as an *in vitro* model. The differentiation status of the cultures was assessed by consecutive TEER measurements over a 21-day differentiation period. TEER values steadily increased until day 15. Thereafter a plateau at approximately 800 Ohm*cm² was reached, which is indicative of a tight epithelial barrier and proper Caco-2 differentiation (figure 1 A). Only cultures that reached at least 700 Ohm*cm² entered subsequent experiments. SEM micrographs showed a homogenous monolayer of microvilli containing cells (figure 1 B). Microvilli are a typical feature of intestinal epithelial morphology and thus confirm Caco-2 differentiation (Lea, 2015). Confocal laser scanning microscopy (CLSM) further confirmed the presence of microvilli and an adequate barrier formation as visualised by immunofluorescence stainings specific for the actin cytoskeleton (magenta, figure 1 C, F) as well as the tight junction proteins ZO-1 (green, figure 1 C, D) and Occludin (red, figure 1 C, E), respectively. In untreated differentiated cultures, the ZO-1 signal appeared closely above or at the same level as the Occludin signal. Nuclei (blue, figure 1 C, G) were always found in the lower most part of the cells (close to the microporous membrane) further confirming cellular polarisation.

Figure 1: Verification of Caco-2 cell differentiation. (A) TEER measurements over the time course of 21 days. Mean values and corresponding standard deviations are from 6 independent experiments with 24 to 60 replicates (d7, 14 and 21) or one single experiment with 12 replicates (d3, 6, 10, 13, 15 and 19) (B) SEM micrograph of the apical surface of a representative Caco-2 mono-culture after 21 days of cultivation, showing a dense coverage with microvilli structures. (C) Representative confocal laser scanning microscopy image of the differentiated Caco-2 mono-culture after 21 days of differentiation and magnifications thereof (D-G). The red square in C indicates the area magnified in D-G. Cells were stained for the tight junction protein ZO-1 (green, C and D), the tight junction protein

Occludin (red, C and E), the cytoskeletal protein F-Actin (magenta, C and F), and nuclei (blue, C and G). Lines and numbers in the schematic drawing of a cell indicate on which z-level the images were taken. Representative images of three independent experiments are shown.

3.3 No ROS overproduction after 2 h of treatment

The formation of ROS was measured as a first stressor in differentiated Caco-2 cultures using the well-established DCF assay (Kucki et al., 2016; May et al., 2018). All 10 tested SAS materials did not induce the formation of ROS up to a concentration of 50 µg/ml after 2 h of treatment. Only the assay specific chemical positive control Sin-1 (50 µM) and the particle positive control MWCNT (20 µg/ml) showed a significant induction compared to the untreated control sample by 7-fold and 3-fold, respectively (figure 2A and supplementary figure S2).

3.4 No influence on cell viability after 48 h of SAS treatment

Cell viability after 24 h and 48 h of treatment with the SAS materials was determined using the resazurin assay. None of the 10 SAS materials induced considerable changes in the viability of differentiated Caco-2 cells up to a concentration of 50 µg/ml and 48 h of treatment (figure 2B and S4). Only for SIPERNAT® 22 S a slight, though statistically significant, reduction in the viability to $83 \pm 8\%$ was detectable after 48 h. However, at all concentrations and time points analysed, mean values were within the fluctuation range of the solvent control, defined as $\pm$ two standard deviations (2s) (supplementary figures S3 and S4). Even in more sensitive non-differentiated Caco-2 cells, no adverse effect of SIPERNAT® 22 S on cell viability was detected (supplementary figure S5 E, F). In contrast, the chemical positive control CdSO₄ reduced cell viability of differentiated Caco-2 cultures significantly and in a dose-

dependent manner after 24 h as well as 48 h (supplementary figures S3 and S4, respectively). The highest concentration (10 mM) reduced cell viability to $7.5 \pm 0.9\%$ after 48 h of treatment (figure 2B). For the particle positive control PS-amine cell viability was significantly reduced to $61.6 \pm 12.3\%$ after 48 h of exposure to 200 $\mu\text{g/ml}$ (figure 2B).

Particle interference reactions and therewith false-positive or false-negative results were excluded for both assays (DCF, resazurin) using a specific cell-free interference control setup (supplementary figure S6 and S7).

Figure 2: Assessment of ROS formation and cell viability. (A) The production of ROS in differentiated Caco-2 cells was measured by the $\text{H}_2\text{-DCF}$ assay after two hours of exposure to 50 $\mu\text{g/ml}$ SAS. 3-Morpholinosydnonimine (Sin-1) at a concentration of 50 μM served as a chemical positive control. Multi-walled carbon nanotubes (MWCNT) at a concentration of 20 $\mu\text{g/ml}$ served as a particle positive control. (B) After incubation of differentiated Caco-2 cells with 50 $\mu\text{g/ml}$ of the indicated SAS for 48 h cell viability was assessed with the resazurin assay. Cadmium sulphate (CdSO_4) at a concentration of 10 mM served as a chemical positive control. Amine modified polystyrene particles

(PS-amine) at a concentration of 200 µg/ml served as a particle positive control. Administered dose of 50 µg/ml SAS corresponds to delivered doses of 16.5 µg/cm² SIPERNAT® 350, 6.6 µg/cm² SIPERNAT® 22, 23.1 µg/cm² SIPERNAT® 22 S, 7.1 µg/cm² SIPERNAT® 2200, 12.3 µg/cm² SIPERNAT® 160, 11.3 µg/cm² SIPERNAT® 50, 21.5 µg/cm² SIPERNAT® 50 S, 5 µg/cm² AEROSIL® OX50, 2.5 µg/cm² AEROSIL® 200 Pharma and 1.4 µg/cm² AEROSIL® 380 F. Mean values and corresponding standard deviations from three independent experiments with four technical replicates each are shown. The dashed orange lines represent the mean values of untreated control samples. *P=0.046, ****P<0.0001 compared to the untreated controls. Full dose-response curves (0 – 50 µg/ml) for all 10 SAS materials can be found in supplementary figures S2 (ROS formation), S3 (viability after 24 h of treatment) and S4 (viability after 48 h of treatment).

3.5 Barrier integrity maintained after 48 h of SAS treatment

To investigate the potential effects of SAS materials on the barrier integrity of differentiated Caco-2 cells, TEER values were measured after 48 h of exposure to a concentration of 50 µg/ml SAS. None of the SAS materials induced significant changes in TEER values compared to the solvent control (figure 3B). Only a one hour treatment with the tight junction disruptor DSS (Gangwar et al., 2017) and the ROS generator Luperox® (Chamoto et al., 2017) at concentrations of 5% and 12.5 to 50 µM, respectively, significantly reduced TEER values below the fluctuation range of the untreated control sample (figure 3A).

Sodium fluorescein translocation studies confirmed the TEER measurements indicating no effect of SAS materials on Caco-2 barrier integrity. No significant changes in the P_{app} compared to the solvent control were measured after treatment with 50 µg/ml SAS for 48 h (figure 3 D). In contrast, treatment with the particulate positive

control PS-amine at a concentration of 200 µg/ml led to an increase in sodium fluorescein translocation (P_{app} $2.0 \pm 1.8 \times 10^{-6}$ cm/s for PS-amine versus P_{app} of $0.3 \pm 0.1 \times 10^{-6}$ cm/s for untreated controls). However, at this concentration cell viability was already impaired (compare figure 2B). Furthermore, microscopic inspection of the Caco-2 monolayer showed the presence of cell-free areas. This indicates rather non-specific effects on barrier integrity due to cell death.

Treatment with the ROS generator Luperox® showed a slight and dose-dependent, though not statistically significant, increase of sodium fluorescein translocation after one hour of treatment compared to the untreated control (figure 3C). DSS did not affect the translocation of sodium fluorescein. These results suggest that TEER measurements are a more sensitive indicator of barrier integrity impairment compared to sodium fluorescein translocation.

Cell-free interference measurements revealed neither auto-fluorescence, nor interference with sodium fluorescein for all SAS materials analysed (supplementary figure S8).

Figure 3: Assessment of the barrier integrity after exposure to SAS. TEER measurements (A, B) and sodium fluorescein translocation studies (C, D) were carried out.

After one hour of parallel incubation with sodium fluorescein and the indicated

concentrations of the positive chemical controls Luper[®] and dextran sulphate sodium salt (DSS) TEER (A) as well as P_{app} (C) measurements were taken. After 48 h of treatment with 50 µg/ml SAS and 200 µg/ml PS-amine TEER values were determined (B). After additional 5 h of sodium fluorescein application, P_{app} values were calculated (D). Administered dose of 50 µg/ml SAS corresponds to delivered doses of
16.5 µg/cm² SIPERNAT[®] 350, 6.6 µg/cm² SIPERNAT[®] 22, 23.1 µg/cm²
SIPERNAT[®] 22 S, 7.1 µg/cm² SIPERNAT[®] 2200, 12.3 µg/cm² SIPERNAT[®] 160,
11.3 µg/cm² SIPERNAT[®] 50, 21.5 µg/cm² SIPERNAT[®] 50 S, 5 µg/cm²
AEROSIL[®] OX50, 2.5 µg/cm² AEROSIL[®] 200 Pharma and 1.4 µg/cm²
AEROSIL[®] 380 F. Mean values and corresponding standard deviations from four independent experiments with two technical replicates each are shown. The mean values of the solvent controls (B, D) or the untreated control samples (A, C) are indicated by a dashed orange line in each graph. Grey dotted lines (A, B) correspond to the standard deviations of the respective means and indicate the range of variability.

**** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$.**

To visualise barrier integrity the tight junction proteins ZO-1 and Occludin were immunofluorescently labelled in differentiated Caco-2 cultures. Additionally, the cytoskeletal protein F-Actin as well as nuclei were counter stained to depict overall cell morphology. Representative images are shown in figure 4. Qualitatively, no changes in any of the markers could be observed after 48 h of treatment with 50 µg/ml SAS. Representative images after AEROSIL[®] 380 F and SIPERNAT[®] 22 S treatment are shown exemplarily in figure 4 (two lower most rows). Besides the already mentioned cell-free areas in the cell monolayer upon PS-amine treatment with 200 µg/ml for 48 h, no specific additional effects on ZO-1 and Occludin signals were noticed. This

observation is in line with the non-specific effects on TEER and sodium fluorescein translocation described above (figure 3). In contrast, treatment with the tight junction disruptor DSS (2%) reduced ZO-1 signal intensity, confirming its effect on TEER values at higher concentrations (5%; figure 3A). While ZO-1 signals appeared above or at the same z-level as Occludin in untreated differentiated Caco-2 cultures (figure 4 and figure 1 D, E), this order was reversed upon 1 h of Luperox® treatment (12.5 µM) (figure 4 second row). F-Actin staining confirmed an otherwise unimpaired cell monolayer in all samples.

Taken together all 10 SAS materials did not influence differentiated Caco-2 cells, neither concerning endpoints of acute cytotoxicity (ROS production and cell viability) nor the epithelium specific endpoint barrier integrity. Therefore, the question arises whether SAS materials are truly inert or "simply" do not get in contact with the cell monolayer.

Figure 4: Barrier integrity evaluations with immunocytochemistry (ICC). Tight junction proteins, indicative of proper barrier formation, were detected by immunofluorescence staining after 1 h of exposure to 12.5 µM Luperox® and 2% dextran sulphate sodium salt (DSS) or after 48 h of exposure to 200 µg/ml PS-amine, 50 µg/ml SIPERNAT® 22 S or AEROSIL® 380 F. The tight junction proteins ZO-1 (green) and Occludin (red), F-Actin (magenta) and nuclei (blue) were stained. Representative images of three independent experiments are shown. Lines and numbers in the schematic drawing of a cell indicate on which z-level the respective images were taken.

3.6 Distinct sedimentation behaviour of different SAS in the dosimetry modelling

To get a first idea of the delivered dose of material and respective deposition rates over 48 h of exposure we made use of the distorted grid (DG) model to simulate nanoparticle dosimetry (DeLoid et al., 2017). Relevant model parameters are summarised in supplementary table S1.

Dosimetry simulations revealed that different fractions of the initially applied mass of 50 µg/ml deposited on the cell surface over time (figure 5). All AEROSIL® agglomerates were smaller and less dense compared to the SIPERNAT® agglomerates (table 1). Accordingly, deposition rates as well as the respective mass fractions that deposited over the timeframe of 48 h were lower for AEROSIL® materials. More than 80% of the two largest agglomerates, SIPERNAT® 22 S and SIPERNAT® 50 S, deposited within the first 3 h and 20 h, respectively. Nearly 90% of the initially applied mass dose of SIPERNAT® 22 S reached the cell surface within the 48 h simulation time. In contrast, AEROSIL® 380 F deposition was slow and only 5.3% were deposited after 48 h (figure 5A and supplementary table S2). For experimental verification, these two extreme cases were investigated further using SEM/EDX and ToF-SIMS techniques. Both methods conduct elemental surface analysis. While SEM/EDX results are purely qualitative, ToF-SIMS allows for the estimation of surface-coverage in a semi-quantitative manner as described in more detail below. To be able to compare *in silico* modelling data with ToF-SIMS results, the deposited fractions were also plotted as "surface area deposited" (cm²/cm²; taking into consideration the theoretical surface area of spherical SAS agglomerates) and "% well bottom occupied" (figure 5B). According to DG modelling results, deposited

617 SIPERNAT® 22 S agglomerates cover approximately four times more of the well
618 bottom area compared to AEROSIL® 380 F agglomerates.

Figure 5: Dosimetry simulations using the DG model. (A) The deposited mass fraction is plotted against the incubation time in hours. (B) For SIPERNAT® 22 S and AEROSIL® 380 F deposited fractions are also given as the deposited surface area per area well bottom (cm^2/cm^2) and the resulting well bottom coverage in %. Non-sticky bottom conditions and an applied SAS concentration of $50 \mu\text{g}/\text{ml}$ were used as input parameters. Further details can be found in supplementary tables S1 and S2.

3.7 SEM/EDX measurements confirm the deposition of SAS onto the cell monolayer

Differentiated Caco-2 cells were treated for 48 h with 50 µg/ml SIPERNAT® 22 S and AEROSIL® 380 F. For both samples, SEM analysis revealed round shaped materials on the cell monolayer on top of the microvilli (figure 6D, G). By visual inspection, differences in the amount and agglomerate size of the deposited material were detectable. SIPERNAT® 22 S agglomerates were smaller but more frequently observed compared to AEROSIL® 380 F of which only very few larger agglomerates were found. Subsequent EDX analysis of the same samples confirmed the presence of silicon in the round shaped objects (figure 6E, F, H, I). No comparable round shaped materials were observed in the solvent control (figure 6A). Accordingly, no silicon was detected in these samples (figure 6B, C) indicating that all silicon peaks resulted from the treatment with SAS materials. Therewith, SEM/EDX confirmed a certain deposition of both SAS samples over time. However, differences in the deposited amount – even though qualitatively seen by visual inspection – cannot be quantified with this method.

Figure 6: SAS detection on differentiated Caco-2 cells by SEM/EDX. (A, D, G) SEM analysis of the apical surface of the differentiated Caco-2 cells after 48 h of treatment with (A) the solvent control (ddH₂O) (D) 50 µg/ml SIPERNAT® 22 S and (G) 50 µg/ml AEROSIL® 380 F. (B, C, E, F, H, I) The same samples were used for subsequent EDX analysis. B, E and H depict the region where the spectra shown in C, F and I were taken, respectively.

3.8 ToF-SIMS analysis confirms distinct deposition behaviour of AEROSIL® 380 F and SIPERNAT ® 22 S

Silicon (Si⁺) spectra were acquired on the apical surface of differentiated Caco-2 cells after 48 h of SIPERNAT® 22 S, AEROSIL® 380 F or ddH₂O treatment. For better statistics, spectra were recorded at five different positions within each sample. Figure [S9A7A](#) displays the full spectrum of the solvent (ddH₂O) treated control sample. Spectra around the nominal mass 28 u containing the fragments Si⁺, CO⁺ and CH₂N⁺ are shown in figure [S97B](#) to D for ddH₂O, SIPERNAT® 22 S and AEROSIL® 380 F treated samples, respectively. A mean normalized intensity for Si⁺ of 209.4 and 60.4

counts was observed for SIPERNAT® 22 S and AEROSIL® 380 F treated samples, respectively, leading to an intensity ratio of 3.5 (figure [7ES9E](#)). The detected Si⁺ content in the solvent control sample did not exceed the noise level of the measurement (figure [S97B](#)), thus confirming that the detected Si⁺ fragments in the SAS treated samples can be attributed to the presence of SAS.

ToF-SIMS is a qualitative measurement technique and the yield of the secondary ions depends on various parameters, but mainly on the amount of the material detected, the matrix effect and charging. Taking into account that all samples had about the same cell coverage on an identical substrate and were prepared in very similar conditions, the matrix effect and charging are expected to be identical for all samples analysed. Consequently, the variation in the count rate of the Si⁺ depends on the amount of silica in the sample.

A quantitative and exact comparison of the Si⁺ concentration in the here assessed samples is difficult, as the CO⁺ fragment (figure [S97 D and E](#)) overlaps slightly with the Si⁺ one, due to the limited mass resolution of the instrument. Regardless of the instrument limitations, the ratio of the Si⁺ intensity of 3.5 is significant enough to show that the exposure to SIPERNAT® 22 S resulted in the higher deposition of silica onto the surface of Caco-2 cells compared to AEROSIL® 380 F exposure, thereby confirming the *in silico* dosimetry results.

Figure 7: Silicon (Si^+) detection on differentiated Caco-2 cells by ToF-SIMS analysis. The deposition of SIPERNAT® 22 S and AEROSIL® 380 F on differentiated Caco-2 cells was assessed after 48 h of incubation with 50 $\mu\text{g/ml}$.

(A) ToF-SIMS spectrum of one position in the solvent control (ddH₂O).

(B, C, D) ToF-SIMS spectra around the nominal mass 28 u (as indicated by the green peak in A) showing the masses of Si^+ , CO^+ and $\text{CH}_2\text{-N}^+$. Full spectra are displayed as inset graphs. Truncated y-axis plots are enlarged to display the variations of the Si^+ count rates in the solvent control (B), the SIPERNAT® 22 S (C) and the AEROSIL® 380 F (D) treated samples, respectively. Each graph contains 5 spectra from 5

~~different positions on the same sample.~~

~~(E) Normalised Si⁺ counts. Box plots comprise values from 8 (SIPERNAT® 22 S) and 10 (AEROSIL® 380 F) individual positions, generated in two independent experiments. The box covers the inner quartile range, whiskers the minimum and maximum values and the centreline represents the median of all values. The intensity ratio of SIPERNAT® 22 S / AEROSIL® 380 F is 3.5.~~

~~****P≤0.0001~~

4 Discussion

Silica is not just silica. Even minor differences in physico-chemical material properties such as primary and agglomerate sizes, crystal structure or surface chemistry and specific surface area (to name only a few) can influence cellular responses (Napierka et al., 2010). A recent study further highlighted that not only the nano-sized fraction of SAS is of toxicological concern but also non-precipitating SAS aggregates > 100 nm could mount an acute cellular response *in vitro* (Murugadoss et al., 2020). Therefore, it is not astonishing, that still in 2018 the EFSA panel on food additives and nutrition sources requested additional toxicity data with the use of "real food-grade E 551 SAS" as well as an improved particle characterisation of the respective materials (EFSA, 2018). Consequently, the present study systematically assessed acute cytotoxic effects of a set of 10 food-grade SAS materials. SAS with low, middle and high specific surface area and from two different production processes (wet and thermal) were used to be able to correlate potential biological effects to these particular material properties. In a first screening approach, we made use of differentiated Caco-2 cells, which are a well-established *in vitro* model of the human intestinal tract. Caco-2 in their

differentiated state have been described to better reflect the *in vivo* situation of the human intestine compared to undifferentiated cells (Pietroiusti et al., 2017). Even though this fact is known, so far only a limited number of studies exists addressing nanomaterial induced effects on differentiated intestinal cells (rather than undifferentiated ones) (Guo et al., 2018, 2017; Sieg et al., 2018; Vila et al., 2018b, 2018a). In particular, the influence of real food-grade SAS materials on differentiated Caco-2 ~~cells~~monocultures, to the best of our knowledge, has only been recently investigated for one SAS material, which did not induce any effects on TEER, barrier morphology or metabolic activity of the cells (Sohal et al., 2020).~~so far not been thoroughly addressed.~~ Other published studies mostly used reference or self-synthesised SiO₂ materials that do not reflect E 551 characteristics (Farcal et al., 2015; Gerloff et al., 2013; Guo et al., 2018; Tarantini et al., 2015; Tsugita et al., 2017; Ye et al., 2017; and summarised by Murugadoss et al., 2017). Furthermore, many *in vitro* studies do not even report details on the particular grade (food-grade or not) of the material used (summarised in Sohal et al., 2018).

Besides characteristics of "as-produced" materials, effects of SAS dissolution during gastrointestinal digestion remains a topic of discussion and research. Recent investigations in simulated gastrointestinal environments (i.e. buffer systems of different pH) revealed that SiO₂ dissolved to a maximum of 65.5% in the gastric phase and the majority of this re-precipitated into larger aggregates when transferred to the intestinal phase (Sohal et al., 2018a). This is in contrast to earlier studies indicating no morphological changes and no degradation of SAS during the gastrointestinal passage (Maier et al., 2013). Based on the latter analyses, which were performed in simulated gastric and fed-state simulated intestinal fluids, SAS materials in the present study were applied without pre-treatment.

In recent years, more complex *in vitro* intestinal models have been developed that include also mucus producing goblet cells as well as uptake competent M-cell like cells (Antunes et al., 2013; des Rieux et al., 2007; Gebert et al., 1996; Hilgendorf et al., 2000; Mahler et al., 2009; Schimpel et al., 2014). These models are considered to be even closer to the *in vivo* situation due to the presence of mucus which has been described as a relevant barrier for drugs (reviewed by Boegh and Nielsen, 2015) but also nanoparticles (e.g. Crater and Carrier, 2010; Liu et al., 2015). ~~It therefore~~The mucus layer represents an important first barrier that is expected to protect the underlying epithelial cells. In several studies, nanomaterials (silver, SiO₂ and CuO NPs) have shown a reduced effect on cell viability and barrier integrity in mucus-producing intestinal co-cultures (Cornu et al., 2020; Saez-Tenorio et al., 2019; Ude et al., 2019, 2017; Vila et al., 2018b) while another study indicates increased sensitivity for ingested nanomaterials (TiO₂, SiO₂, ZnO and Fe₂O₃) in such a co-culture model (Sohal et al., 2020). The most obvious disadvantage, however, is the complexity of the systems that precludes the fast screening of numerous materials. Since the mucus barrier is lacking in the differentiated Caco-2 mono-culture our study mimics a worst-case scenario in which the applied material gets in direct contact with the cell type of interest. Therefore, we propose the differentiated Caco-2 model as a valuable approach for the pre-screening of food-relevant materials and to obtain specific insights on their impact on the most abundant cell type of the intestine. This could help to identify potential toxic materials that should be further investigated in more advanced intestinal co-culture models. A tiered approach is required to overcome the limitations of the differentiated Caco-2 model especially regarding the absence of a mucus layer and other relevant intestinal cell types. Therefore, our model perfectly combines the *in vivo* relevance of differentiated cells with reasonable sensitivity and throughput.

779 ~~Accordingly~~ Furthermore, we aimed to apply physiologically relevant dose ranges of
780 ~~0.02-11 $\mu\text{g}/\text{cm}^2$ per person per day (Sohal et al., (2018b) and explanations in the~~
781 ~~introduction).~~, with applied SAS concentrations of 3 to 50 $\mu\text{g}/\text{ml}$ we aimed at
782 ~~physiologically relevant dose ranges.~~ Assuming 100% deposition, ~~applied doses of 3-~~
783 ~~50 $\mu\text{g}/\text{ml}$ this would~~ corresponds to 1.6 $\mu\text{g}/\text{cm}^2$ - 26.2 $\mu\text{g}/\text{cm}^2$, ~~and therewith indeed~~
784 ~~covers realistic doses (Sohal, 2018; and explanations in the introduction) up to a slight~~
785 ~~overdose situation at the highest concentration analysed. However,~~ Taking further the
786 *in silico* dosimetry ~~calculations suggest considerable differences in deposited fractions~~
787 ~~results~~ for the different materials ~~into consideration~~ (supplementary table S2), ~~even~~
788 ~~F~~for the weakest “depositer” AEROSIL® 380 F, ~~the~~ ~~we achieved a~~ computed realistic
789 dose-range ~~of is~~ 0.1 -1.4 $\mu\text{g}/\text{cm}^2$ ~~while for the highest “depositer” SIPERNAT® 22 S it~~
790 ~~is 1.4 -23.1 $\mu\text{g}/\text{cm}^2$, thus deposited doses close to estimated realistic doses were~~
791 ~~included in our *in vitro* toxicity assessment.~~ Although the deposited mass fraction of
792 SIPERNAT® 22 S was calculated to be about 16 times higher compared to the one of
793 AEROSIL® 380 F (figure 5A and supplementary table S2), the cell surface area that is
794 covered by these amounts differed only by a factor of ~4 (figure 5B, figure ~~S97~~E and
795 supplementary table S2). ~~It can only be speculated which parameter (weight % or~~
796 ~~surface area occupied) might have a greater impact on cellular functionality.~~ Moreover,
797 the specific surface area of a material has been reported to be a relevant characteristic
798 in terms of toxicity (e.g. Oberdörster et al., 2005; Schmid and Stoeger, 2016). With
799 around 400 m^2/g , AEROSIL® 380 F possesses an approximately twice as large
800 specific surface area compared to SIPERNAT® 22 S (~200 m^2/g).

801 ~~Recently, the silanol content of SAS (non food-grade) has been inversely associated~~
802 ~~with cell membrane damage, reduced cell viability, acellular free radical generation in~~
803 ~~RAW264.7 macrophages but not in normal small airway epithelial cells (SAEC) (Rubio~~
804 ~~et al., 2019).~~ In general, silanol numbers per square nanometer of SAS surface are

higher for precipitated than pyrogenic SAS forms (Fruijtier-Pölloth, 2016), thus differences in silanol content can also be expected for the here investigated SAS materials. How is this perceived by the differentiated Caco-2 cell monolayer? However, despite the use of a broad panel of different food-relevant SAS with considerable differences in particle characteristics, we could not identify any adverse effects of SAS on differentiated Caco-2 cells. The absence of a measurable biological response was due to the inertness of the materials rather than a dosimetry artefact as we confirmed the deposition of SAS materials by dosimetry simulations and experimentally by SEM/EDX and ToF-SIMS for the particles with highest and lowest deposition. One explanation for the lack of previously described structure-activity relationships in our study could be that SAS effects are highly cell type-specific with immune cells (macrophages, dendritic cells) being more sensitive (Rubio et al., 2019; Winkler et al., 2017) than epithelial cells (Rubio et al., 2019 and our own data).

Since no biological effect could be observed for any of the SAS materials analysed, a conclusive direct correlation of particular physico-chemical material properties and a specific biological response was not possible. Nevertheless, our results still imply that neither the production process, the specific surface area, nor aggregate and agglomerate size are of relevance for *in vitro* toxicity in the differentiated Caco-2 model. Beyond that, the confirmed deposition of SAS materials indicates that the absence of a measurable biological effect was due to the inertness of the materials rather than a dosimetry artefact. Even Only for SIPERNAT® 22 S, which showed the highest deposition of almost 90% of the applied dose, only a minor small adverse effect on but significant reduction of the viability (down to 83%) of differentiated Caco-2 cells at the highest concentration (50 µg/ml; 23 µg/cm²) was observed after 48 h of exposure whereas effects on ROS generation and disruption of barrier integrity were absent. According to ISO 10993-5 (INTERNATIONAL STANDARD, 2009), this is not of

biological relevance since only a reduction in cell viability down to 70% is considered a substantial cytotoxic effect. In addition, taking the above consumption patterns and calculations into consideration, the A deposited dose of 23 $\mu\text{g}/\text{cm}^2$ probably represent a non-realistic acute exposure since it exceeds the current worst-case exposure scenario of 11 $\mu\text{g}/\text{cm}^2$ by a factor of two. Nevertheless, we further examined the effects of SIPERNAT® 22 S in undifferentiated Caco-2 cultures that lack a mature brush border and are in general more susceptible than differentiated Caco-2. However, viability of undifferentiated Caco-2 was not affected by the particles even at high doses up to 50 $\mu\text{g}/\text{ml}$. It is possible that SIPERNAT® 22 S might have some biological effects on intestinal cells and this should be examined in more detail in future studies using more advanced intestinal co-cultures and including additional functional endpoints. Furthermore, since the same concentration of these particles did not reduce the viability of more vulnerable undifferentiated Caco-2 cultures and did not induce ROS production or disrupt barrier integrity, we conclude that the observed reduction in viability is not of considerable biological relevance.

5 Conclusion

Due to their anti-caking properties, SAS materials have been used in different food products for more than half a century (Maynard, 2014). However, for regulatory purposes, comprehensive toxicological studies on real food-grade materials are still limited (EFSA, 2018).

Here we presented a precautionary *in vitro* screening approach using a panel of 10 well-characterised food-grade SAS materials and differentiated Caco-2 cells. Verification of the direct SAS-cell contact, the realistic and *in vivo* relevant dose range as well as thoroughly implemented assay and nanomaterial controls confirm that the *in vitro* system is viable and able to detect potential adverse effects. Nevertheless, independent of the production process and the specific surface area, none of the 10 SAS materials induced a biological response.

~~Based on this first systematic screening approach and its~~ Despite the reassuring outcome in terms of ~~consumer safety~~ absence of acute intestinal epithelial barrier toxicity, further investigations in more sophisticated co-culture barrier models should be considered. These should not only include mucus, as an important additional barrier but also take into consideration the potential bio-transformation of SAS materials under *in vivo* relevant digestion conditions. Furthermore, intestinal specific endpoints should be investigated to better examine the effect on the intestinal function as well as long-term studies should address chronic exposure scenarios, ~~taking into account also~~ sublethal effects.

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882 **Conflict of interest**

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887

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Figure S1: Representative TEM images used to determine primary structure sizes as well as aggregate sizes of SAS materials. SIPERNAT® 22 S (A, B) and AEROSIL® 380 F (C, D) are shown exemplarily.

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Figure S2: Concentration dependent analysis of ROS formation upon SAS treatment for 2 h. The production of ROS in differentiated Caco-2 cells was measured by the DCF assay after two hours of exposure to the indicated concentrations of the 10 different SAS materials. None of the 10 SAS materials analysed produced ROS. 3-Morpholinosydnonimine (Sin-1) served as a chemical positive control and induced a 7-fold increase at a concentration of 50 μM. Multi-walled carbon nanotubes (MWCNT) served as a particle positive control inducing a dose depend increase up to 3-fold at a concentration of 20 μg/ml. Mean values and corresponding standard deviations from three independent experiments with four technical replicates each are shown. The orange dashed line indicates the 100% level of the untreated control sample. **** $P \leq 0.0001$

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Figure S3: Concentration dependent analysis of cell viability of differentiated Caco-2 cells after 24 h of SAS exposure. Following the incubation of differentiated Caco-2 cells with various concentrations of different SAS for 24 h, cell viability was assessed with resazurin. (A) Cadmium sulphate (CdSO_4) served as a chemical positive control and induced a dose-dependent decrease in cell viability. (B) Amine modified polystyrene particles (PS-amine) served as a particle positive control. (C) SIPERNAT® 350. (D) SIPERNAT® 22. (E) SIPERNAT® 22 S. (F) SIPERNAT® 2200. (G) SIPERNAT® 160. (H) SIPERNAT® 50. (I) SIPERNAT® 50 S. (J) AEROSIL® OX50. (K) AEROSIL® 200 Pharma. (L) AEROSIL® 380 F. Shown are administered doses of SAS and the fraction deposited are 63% for SIPERNAT® 350, 25% for SIPERNAT® 22, 88% for SIPERNAT® 22 S, 27% for

SIPERNAT® 2200, 47% for SIPERNAT® 160, 43% for SIPERNAT® 50, 82% for SIPERNAT® 50 S, 19% for AEROSIL® OX50, 9.5% for AEROSIL® 200 Pharma and 5.3% for AEROSIL® 380 F. Mean values and corresponding standard deviations from three independent experiments with four technical replicates each are shown. The orange dashed line and the grey dotted lines resemble the mean value of the solvent control sample and twice the corresponding standard deviations, respectively. All values therein are within the range of solvent treated sample variation and are thus considered without effect.

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Figure S4: Concentration dependent analysis of cell viability of differentiated Caco-2 cells after 48 h of SAS exposure. Following the incubation of differentiated Caco-2 cells with various concentrations of different SAS for 48 h, cell viability was assessed with resazurin. (A) CdSO_4 served as a chemical positive control and induced a dose-dependent decrease in the cell viability. (B) PS-amine served as a particle positive control and decreased viability at a concentration of 200 $\mu\text{g/ml}$. (C) SIPERNAT® 350. (D) SIPERNAT® 22. (E) SIPERNAT® 22 S. (F) SIPERNAT® 2200. (G) SIPERNAT® 160. (H) SIPERNAT® 50. (I) SIPERNAT® 50 S. (J) AEROSIL® OX50. (K) AEROSIL® 200 Pharma. (L) AEROSIL® 380 F. Shown are administered doses of SAS and the fraction deposited are 63% for SIPERNAT® 350, 25% for SIPERNAT® 22, 88% for SIPERNAT® 22 S, 27% for

SIPERNAT® 2200, 47% for SIPERNAT® 160, 43% for SIPERNAT® 50, 82% for SIPERNAT® 50 S, 19% for AEROSIL® OX50, 9.5% for AEROSIL® 20 Pharma and 5.3% for AEROSIL® 380 F. Mean values and corresponding standard deviations from three independent experiments with four technical replicates each are shown. The orange dashed line and the grey dotted lines resemble the mean value of the solvent control sample and twice the corresponding standard deviations, respectively. All values therein are within the range of solvent treated sample variation and are thus considered without effect.

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1241 Resazurin assay in undifferentiated Caco-2 cells

1242 Caco-2 cells were seeded into 96-well plates at a density of 4.5×10^5 cells per well in a
1243 total volume of 200 µl complete cell culture medium. Cells were allowed to attach for
1244 48 h. Treatment volumes in the 96-well plates were calculated to match medium
1245 heights of 5.2 mm to achieve identical dosing conditions compared to the differentiated
1246 Caco-2 cells in the HTS 96-well inserts. This resulted in an actual treatment volume of
1247 169 µl for the undifferentiated Caco-2 cells. Cells were treated with concentrations of
1248 0, 3.125, 6.25, 12.5, 25 and 50 µg/ml SIPERNAT® 22 S for 24 and 48 h in fresh
1249 complete phenol red free cell culture medium. CdSO₄ was used in concentrations of 0,
1250 1, 10, 100, 1000, and 10,000 µM and served as a chemical positive control. PS-amine
1251 in concentrations of 0, 12.5, 25, 50, 100, and 200 µg/ml served as a particle positive
1252 control. All 0 µg/ml samples received an equivalent volume of ddH₂O in medium as
1253 solvent control. The resazurin stock solution (1 mg/ml in ddH₂O) was prepared freshly
1254 for every experiment. 24 and 48 h post-exposure 22.5 µL of a 21.25 µg/ml pre-dilution
1255 of resazurin in PBS were added directly to each well resulting in a final concentration
1256 of 2.5 µg/ml resazurin in complete phenol red free cell culture medium. The cells were
1257 incubated for 2 h at standard growth conditions and the fluorescence was measured
1258 with a multi-well plate reader (Mithras², Berthold Technologies) at 530/585 nm

(excitation/emission). Data shown represent the mean and corresponding standard deviations of three independent experiments with four technical replicates each.

As shown in figure S5 SIPERNAT® 22 S did not affect viability of undifferentiated Caco-2 cells up to 48 h of treatment and a concentration of 50 µg/ml. Values of all concentrations analysed were within the range of solvent treated sample variation indicated by the dotted grey lines which resemble twice the standard deviation of the solvent control sample. In contrast treatment of undifferentiated Caco-2 cells with the positive control particle PS-amine resulted in a dose-dependent decrease in cell viability after 24 (blue squares) as well as 48 h (red dots) (figure S5C, D, respectively). This confirms the reported higher sensitivity towards toxic nanoparticle insult of undifferentiated Caco-2 cells compared to differentiated Caco-2 cells (Gerloff et al., 2013; Pietroiusti et al., 2017; Song et al., 2015). Similarly, the chemical positive control CdSO₄ induced a dose-dependent decrease in cell viability both after 24 and 48 h of treatment (figure S5A, B, respectively).

Figure S5: Concentration dependent analysis of cell viability of undifferentiated Caco-2 cells after 24 h (blue squares, A, C, E) and 48 h (red circles, B, D, F) of exposure. Following the incubation of undifferentiated Caco-2 cells for 24 h (blue squares) and 48 h (red circles) cell viability was assessed with resazurin. (A, B) The chemical positive control CdSO₄ induced a dose-dependent decrease in cell viability at both time points. (C, D) Similarly, the particle positive control PS-amine induced a dose-dependent reduction in cell viability at both time points. (E, F) SIPERNAT® 22 S did not influence cell viability at both time points and up to a concentration of 50 μ g/ml. Shown are administered doses of SIPERNAT® 22 S and the fraction deposited is 88% of the administered dose. The orange dashed line and the grey dotted lines resemble the mean value of the solvent control sample and twice the corresponding standard deviations, respectively. All values therein are within the range of untreated sample variation and are thus considered without effect.

Interference assessment in a cell-free environment

Different types of nanomaterials have been shown to interfere with absorbance as well as fluorescence based *in vitro* assays (Belyanskaya et al., 2007; Casey et al., 2007; Hirsch et al., 2011; Monteiro-Riviere et al., 2009). To avoid false positive or false negative results it is thus important to address potential interference reactions with the respective assays used. We established cell free interference controls for all assays that were run independently of and in parallel or prior to the cell based assays.

Interference assessment in the DCF assay

Oxidation of H₂DCF through SAS interaction, quenching of an existing fluorescence signal due to the presence of SAS and SAS auto-fluorescence were analysed in a cell free environment. A detailed description of the interference detection method has been published previously by May et al., (2018).

To assess the intrinsic reactivity (or oxidative potential) the de-acetylated H₂DCF molecule was incubated for 2 h with increasing concentrations of SAS as well as the positive controls Sin-1 and MWCNT. As shown in figure S6A the chemical positive control Sin-1 (50 µM) is able to process the H₂DCF molecule in a cell free environment leading to a 27-fold increase in relative fluorescence compared to the untreated sample. Similarly, 20 µg/ml of the particle positive control MWCNT induced a 29-fold increase in relative fluorescence. In contrast, none of the ten SAS materials influenced H₂DCF fluorescence in a cell free environment up to a concentration of 50 µg/ml and 2 h of incubation.

Quenching effects were analysed using the fluorescent molecule DCF (Sigma, Buchs, Switzerland). No quenching of the DCF signal was observed for any of the ten SAS materials up to a concentration of 50 µg/ml and 2 h of incubation (figure 6B).

1301 Auto-fluorescence of increasing SAS concentrations were measured in HBSS. None
 1302 of the ten SAS materials exhibited an auto-fluorescence signal (figure S6C) after 2 h
 1303 of incubation and up to a concentration of 50 µg/ml.
 1304 All measurements were carried out at a wavelength of 485/528 nm
 1305 (excitation/emission) using a multimode plate reader (Mithras², Berthold
 1306 Technologies).
 1307 Data shown in figure S6 represent the mean values and corresponding standard
 1308 deviations from three independent experiments with four technical replicates each.

Figure S6: Interference assessment of SAS with the DCF assay. (A) Assessment of the intrinsic reactivity. The chemical control (Sin-1) as well as the particle positive control (MWCNT) increased the H₂DCF fluorescence signal after 2 h of incubation. All 10 SAS materials did not process H₂DCF in a cell free environment. The orange dotted line represents the mean value of the solvent control to which all values were normalized. (B) Assessment of quenching reactions. SAS did not quench an existing DCF signal. (C) Assessment of auto-fluorescence. SAS did not show any sign of auto-fluorescence. The

orange dotted lines in B and C represent the solvent control of the DCF signal to which all values were normalized. Mean values and corresponding standard deviations from three independent experiments with four technical replicates each are shown.

Interference assessment in the resazurin assay

In order to assess potential interference with the resazurin assay three different parameters were investigated: quenching of an existing resorufin signal, reduction of resazurin to resorufin by SAS and auto-fluorescence of SAS materials. The treatment of the interference samples and the assay procedure were identical to the protocol described in the materials and methods section of the main manuscript but were carried out in 96-well cell culture plates (as opposed to HTS 96-well inserts). To ensure identical dosimetry conditions, volumes were adapted accordingly. Serial dilutions of SAS (0, 3.125, 6.25, 12.5, 25 and 50 µg/ml) were prepared in complete phenol red free cell culture medium and 169 µl were added to the respective wells of a 96-well plate. To determine potential quenching effects of SAS 22.5 µl of the fluorescent molecule resorufin were added to each well containing the respective SAS dilutions. Likewise, to assess the reduction of resazurin to resorufin, 22.5 µl resazurin were added to the respective wells. Auto-fluorescence of SAS was assessed upon addition of 22.5 µl PBS. All samples were incubated for 2 h under standard growth conditions and fluorescence was measured at a wavelength of 530/585 nm (excitation/emission) using a multimode plate reader (Mithras², Berthold Technologies). None of the ten SAS quenched the resorufin signal, reduced resazurin to resorufin or exhibited auto-fluorescence up to a concentration of 50 µg/ml (figure S7).

Figure S7: Interference assessment of SAS with the resazurin assay. (A) SAS did not quench an existing resorufin signal. (B) SAS did not process resazurin in a cell free environment. (C) SAS did not show any sign of autofluorescence. Mean values and corresponding standard deviations from three independent experiments with two technical replicates each are shown. All values were normalized to the solvent control of resorufin which is shown as an orange dashed line in all graphs.

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1331 Interference assessment in sodium fluorescein translocation studies

1332 Two parameters of potential SAS interference were determined: quenching of the
1333 sodium fluorescein fluorescence signal and the auto-fluorescence of SAS. Three
1334 different concentrations of SAS were assessed: 0.5, 5 and 50 µg/ml. SAS materials
1335 were pre-incubated with 10% FCS for 48 h prior to the interference assessment.

1336 Therefore, the pre-incubated SAS materials were either diluted in complete phenol red
1337 free cell culture medium containing 5 µM sodium fluorescein or complete phenol red
1338 free cell culture medium only to assess quenching effects and auto-fluorescence,
1339 respectively.

1340 100 μ l of each dilution were added in duplicates to a 96-well plate and incubated for
 1341 5 h under standard growth conditions. Fluorescence was measured at a wavelength of
 1342 460/515 nm (excitation/emission) using a multimode plate reader (Mithras², Berthold
 1343 Technologies). Neither quenching of the sodium fluorescein signal nor auto-
 1344 fluorescence was detected in the presence of up to 50 μ g/ml SAS.
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Figure S8: Interference assessment of SAS with the sodium fluorescein translocation assay. (A) SAS did not quench an existing signal of sodium fluorescein. (B) SAS materials were not auto-fluorescent. Mean values and corresponding standard deviations from three independent experiments with two technical replicates each are shown. All values were normalized to the solvent control of sodium fluorescein which is shown as an orange dashed line in both graphs.

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Figure S9: Silicon (Si^+) detection on differentiated *Caco-2* cells by ToF-SIMS analysis. The deposition of SIPERNAT® 22 S and AEROSIL® 380 F on differentiated *Caco-2* cells was assessed after 48 h of incubation with 50 $\mu\text{g}/\text{ml}$.

(A) ToF-SIMS spectrum of one position in the solvent control (ddH_2O). (B, C, D) ToF-SIMS spectra around the nominal mass 28 u (as indicated by the green peak in A) showing the masses of Si^+ , CO^+ and $\text{CH}_2\text{-N}^+$. Full spectra are displayed as inset graphs. Truncated y-axis plots are enlarged to display the variations of the Si^+ count rates in the solvent control (B), the SIPERNAT® 22 S (C) and the AEROSIL® 380 F (D) treated samples, respectively. Each graph contains 5 spectra from 5 different positions on the same sample. (E) Normalised Si^+ counts. Box plots comprise values from 8 (SIPERNAT® 22 S) and 10 (AEROSIL® 380 F) individual positions, generated in two

1376 *independent experiments. The box covers the inner quartile range, whiskers the minimum and*
1377 *maximum values and the centreline represents the median of all values. The intensity ratio of*
1378 *SIPERNAT® 22 S / AEROSIL® 380 F is 3.5. **** $P \leq 0.0001$*

1379

	Property	Value	Unit
solvent properties	viscosity	0.00081	Pa s
	density	1.0104	g/cm ³
	temperature	37	°C
particle properties	density	2.285	g/cm ³
	particle diameter size distribution	compare DLS measurements	nm
	fractions per bin (=volume weighted fractions)	compare DLS data	
	agglomerate effective density	compare VCM results	g/cm ³
experimental parameters	height of suspension	5.24	mm
	initial total SAS concentration	0.05	mg/cm ³
	total time of simulation	48	h
	sedimentation force	1	g
model parameters	height of subcompartment	0.005	mm
	time interval for simulation	0.5	s
output parameters	data output time interval	60	min
	output compartment height	0.01	mm
	plot or not plot	1	
	write bottom output only (1) or all data (0)	1	
advanced model parameters			
concentration dependence of transport parameters	sedimentation coefficient	0	
	concentration dependence ks		
	diffusion coefficient concentration dependence kd	0	
dissolution parameter	initial dissolution fraction (at t0)	0	
	method for modelling dynamic dissolution in terms of agglomerates	1	
	type of dissolution rate	0	
	times for dissolution fraction data (h)		
	dissolution fractions corresponding to specified times		
"stickiness"	particle-cell adsorption parameters	0	
	fraction of free particles/agglomerates (in bottom compartment) that bind per second		
	adsorption dissociation constant for agglomerate binding to cells (K_D)	1x10 ⁻⁹	mol/L

Table S1: Parameters used for the DG simulations. Particle specific parameters are depicted in blue. Respective agglomerate effective density values for each particle type are summarized in table 1 (main text). Particle size distributions ("DLS measurements") are given in supplementary file 2.

Material	initially applied concentration (µg/ml)	fraction deposited (%)	resulting delivered dose (µg/cm ²)	resulting well surface occupied (%)
SIPERNAT® 350	50	63	16.5	11.8
SIPERNAT® 22	50	25	6.6	10.3
SIPERNAT® 22 S	50	88	23.1	5.4
SIPERNAT® 2200	50	27	7.1	6.9
SIPERNAT® 160	50	47	12.3	7.6
SIPERNAT® 50	50	43	11.3	10
SIPERNAT® 50 S	50	82	21.5	5.5
AEROSIL® OX50	50	19	5	6.1
AEROSIL® 200 Pharma	50	9.5	2.5	2
AEROSIL® 380 F	50	5.3	1.4	1.5

1385 **Table S2:** Results of the DG model.

A **B**

A

B

C

D

H₂O

50 µg/ml
SIPERNAT®
22 S

50 µg/ml
AEROSIL®
380 F

Material	Code ^I	Production process	Primary structure size/ nm ^{II}	Aggregate size (ECD)/ nm ^{II}	Specific surface area/ (m ² /g) ^{III}	Material density/ (g/cm ³) ^{IV}	Agglomerate effective density/ (g/cm ³) ^V	Agglomerate size/ nm ^{VI}	Point of zero charge/ pH ^{VII}	Zeta potential/ mV ^{VIII}
SIPERNAT® 350		precipitated	30.3 ± 6.8	276.3	55	2.15	1.33	623 ± 15	1.7	n.a.
SIPERNAT® 22	P-5	precipitated	10 ± 2.6	82.2	180	2.12	1.65	325 ± 37	1.7	-37.1
SIPERNAT® 22 S		precipitated	10 ± 2.6	82.2	185	2.14	1.20	743 ± 38	2.0	-37.1
SIPERNAT® 2200		precipitated	9 ± 1.4	44.9	185	2.12	1.25	324 ± 13	1.5	n.a.
SIPERNAT® 160	P-1	precipitated	12.2 ± 2.7	58.3	180	2.16	1.18	555 ± 32	1.7	-41.2
SIPERNAT® 50	P-2	precipitated	3.1 ± 0.7	59.8	460	2.12	1.15	645 ± 79	1.7	-13.7
SIPERNAT® 50 S		precipitated	3.1 ± 0.7	59.8	460	2.12	1.16	747 ± 86	1.7	-13.7
AEROSIL® OX50	F-1	fumed	41.4 ± 18.3	233.7	45	2.32	1.25	299 ± 6	2.3	-34.0
AEROSIL® 200 Pharma		fumed	7.8 ± 1.8	161.1	210	2.38	1.07	294 ± 5	2.3	-11.8
AEROSIL® 380 F	F-3	fumed	8 ± 2.7	101.9	390	2.29	1.09	318 ± 15	2.4	-10.6

Table 1: Properties of food-grade SAS materials. ^IThe same materials were used in a recent study by Wiemann and co-workers (Wiemann, 2019) under the respective codes given here. ^{II}Analyzed by TEM. The arithmetic mean and corresponding standard deviations from 2000 and 1000 items are given for primary structure and aggregate sizes, respectively. ECD: equivalent circular diameter. ^{III}Assessed by N₂-BET. ^{IV}Assessed by gas displacement pycnometry. ^VAssessed by volumetric centrifugation method from 0.1 mg/ml suspensions in ddH₂O. ^{VI}Z-average values as assessed by DLS from 0.05 mg/ml suspension in ddH₂O. The mean and corresponding standard deviations from two independent measurements with three technical replicates each are given. ^{VII/VIII}Point of zero charge as well as zeta potential were measured with an electroacoustic sensor at a solid density of 2.1 g/ml.

Declaration of interests

□ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

□The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

As detailed in each coi_disclosure document the following financial interests are declared:
Ms. Hempt reports grants from CCMX, grants and non-financial support from Evonik Resource Efficiency GmbH, during the conduct of the study.
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