Survey of Food-grade Silica Dioxide Nanomaterial Occurrence, Characterization, Human Gut Impacts and Fate across Its Lifecycle

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ABSTRACT

There is increasing recognition of the importance of transformations in nanomaterials across their lifecycle, yet few quantitative examples exist. We examined food-grade silicon dioxide (SiO₂) nanomaterials from its source (bulk material providers), occurrence in food products, impacts on human gastrointestinal tract during consumption, and fate at wastewater treatment plants. Based upon XRD, XPS and TEM analysis, pure SiO₂ present in multiple food-grade stock SiO₂ exhibited consistent morphologies as agglomerates, ranging in size from below 100 nm to > 500 nm, with all primary particle size in the range of 9–26 nm and were most likely amorphous SiO₂ based upon high resolution TEM. Ten of 14 targeted foods purchased in the USA contained SiO₂ of the same morphology and size as the pristine bulk food-grade SiO₂, at levels of 2 to 200 mg Si per serving size. A dissolution study of pristine SiO₂ showed up to 7% of the dissolution of the silica, but the un-dissolved SiO₂ maintained the same morphology as the pristine SiO₂. Across a realistic exposure range, pristine SiO₂ exhibited adverse dose-response relationships on a cell model (microvilli) of the human gastro-intestinal tract, association onto microvilli and evidence that SiO₂ lead to production of reactive oxygen species (ROS). We also observed accumulation of amorphous nano SiO₂ on bioflocs in tests using lab-cultured activated sludge and sewage sludges from a full-scale wastewater treatment plant (WWTP). Nano-scale SiO₂ of the same size and morphology as pristine food-grade SiO₂ was observed in raw sewage at a WWTP, but we identified non-agglomerated individual SiO₂ particles with an average diameter of 21.5 ± 4.7 nm in treated effluent from the WWTP. This study demonstrates an approach to track nanomaterials from source-to-sink and establishes a baseline occurrence of nano-scale SiO₂ in foods and WWTPs.

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KEYWORDS: food, silica, silicon, nanotechnology, nanoparticle, sewage, additive

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TOC/Abstract Art

A Survey of Food-grade Nano SiO₂ across Its Lifecycle

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1. Introduction

Research gaps exist related to the occurrence, transformations and safety of engineered nanomaterials across various components of their life cycle (i.e., source-to-sink) from synthesis, through use, and to end-of-life (NNI, 2014). Viewing nanomaterials with this life cycle perspective differs from a life cycle analysis, the latter of which is a tool for evaluating a product's environmental impacts through all stages of its life cycle. Food additives have emerged as a major use of engineered nanomaterials, yet recent reviews indicate we know little about their effects, their ability to undergo transformations, or the ability to characterize them in complex matrices (Canady and Tsytiskova, 2013; Singh et al., 2014; Szakal et al., 2014; Yada et al., 2014). Silicon dioxide (i.e., SiO_2) has the highest production volume of all engineered nanomaterials, and its use as a food additive is a major sector (Keller and Lazareva, 2014; Keller et al., 2013b; Lazareva and Keller, 2014). SiO_2 is widely used in foods and other products as a defoaming agent/stabilizer, adsorbent, carrier, conditioning agent, chill-proofing agent, filter aid, emulsifying agent, viscosity control agent, anti-settling agent, and anti-caking agent (Lewis & Harrison and Washington, 2010; OECD, 2004). The United States Food and Drug Administration (USFDA) and the Code of Federal Regulations Title 21 permit the use of SiO_2 in foods not to exceed 2% by weight (U.S.FDA, 2014). The United Kingdom Food Standards Agency permits the use of SiO_2 in foods as anti-caking agent (E551) (FSA, 2010), and it can be added to a broad variety of foods up to 5% by weight in European markets (Lewis & Harrison and Washington, 2010). Commonly used food-grade SiO_2 products include synthetic amorphous silica (SAS), sodium aluminum silicates, and calcium silicates (Lewis & Harrison and Washington, 2010; OECD, 2004). Food-grade SiO_2 is produced either thermally (e.g., fumed SiO_2) or through wet chemistry (e.g., SiO_2 gel and precipitated SiO_2) (OECD, 2004). The worldwide production

of SiO₂ in 2000 was 432,900 tons, with ~ 90% reported to be SAS for food industry and other sectors (OECD, 2004). The production, use, and end-of-life disposal of food-grade SiO₂ can lead to direct and indirect exposure of humans and the environment (Hansen et al., 1999;van Kesteren et al., 2014), and there are emerging concerns about its potential risk to humans and ecosystems (Maynard, 2014).

The toxicity of food-grade SiO₂ has been evaluated *in vitro* and *in vivo* (ECETOC, 2006;OECD, 2004). The European Center for Ecotoxicology and Toxicology of Chemicals and the Organization for Economic Cooperation and Development found no acute toxicity of amorphous SiO₂ after conducting a toxicity study on rats through oral and dermal exposure, with the highest exposure concentration of 20 g SiO₂/kg body weight (ECETOC, 2006;OECD, 2004). No significant intestinal absorption of amorphous SiO₂ was observed in animals or humans (OECD, 2004). However, a recent study showed that fumed SiO₂, a form of SAS, produced reactive oxygen species (ROS) and caused red blood cell hemolysis (heparinized mouse cell) (Zhang et al., 2012). Other work suggests the impact of amorphous SiO₂ on humans, especially on the gastrointestinal tract, should be re-evaluated because there are significant differences in physiology and nutrient uptake of the gastrointestinal tract between humans and rats (Faust et al., 2014a;Maynard, 2014).Additionally, nano-sized SiO₂ (i.e., nano SiO₂) particles can exist in intestine digestion stage even though they would agglomerate under acidic condition during the saliva and gastric digestion stages (Peters et al., 2012).Thus it is necessary to explore the impact of food-grade SiO₂ on microvilli, as the brush border cytoskeleton is more fragile in humans than rodents (Carboni et al., 1987).

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10 116 (Keller et al., 2013a; Piccinno et al., 2012; Som et al., 2011). Material flow modeling estimates
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12 117 that approximately 7,000 tons/year of nano SiO₂ (including food-grade nano SiO₂) enters
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15 118 wastewater treatment plants (WWTPs) (Keller et al., 2013a). Slight removal of uncoated nano
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17 119 SiO₂ particles occurs in WWTPs, either by coagulation using poly aluminum chloride or by the
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20 120 activated sludge process (Chang et al., 2007; Jarvie et al., 2009). These previous studies focus on
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22 121 well-dispersed spherical nano SiO₂ particles (Chang et al., 2007; Jarvie et al., 2009), but the
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24 122 removal of food-grade SiO₂ nanomaterial (e.g., fumed nano SiO₂) in the activated sludge process
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27 123 has not yet been fully explored. Experimental data on removal of food-grade SiO₂ is needed to
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30 124 understand its fate across the food-system and discharge into the environment (i.e., a life-cycle
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38 127 The objective of this research was to understand occurrence, characterization and
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41 128 transformations of food-grade nano SiO₂, a high volume engineered nanomaterial with direct
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43 129 human and environmental contact. First we characterized six food-grade SiO₂ bulk material
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48 131 transmission electron microscopy [TEM]), hydrodynamic diameter and zeta (ζ) potential (using
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51 132 Phase Analysis Light Scattering [PALS]), crystal structure (using X-ray diffraction [XRD]), and
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53 133 surface functionality (using X-ray photoelectron spectroscopy [XPS]). Second, we quantified the
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ingredients. Third, as an indicator of a process leading to transformation of food-grade nano SiO₂, we tested dissolution potential of bulk food-grade SiO₂ in ultrapure water, tap water, 1 mM of sodium bicarbonate (NaHCO₃), and a culture medium. Fourth, we quantified the impact of food-grade SiO₂ in culture media on microvilli using a Caco-2BBel cell system, which is an accepted model of the human intestine (Peterson et al., 1993; Peterson and Mooseker, 1993), and we hypothesized that the amorphous food-grade nano SiO₂ could lead to the disruption of microvilli surface structure. Finally, we investigated occurrence and structural properties of bulk food-grade SiO₂ in lab-scale tests and prospected the presence of nano SiO₂ in sludge samples from a local WWTP to determine whether SiO₂ is present in treated effluent that is discharged to surface waters or in sewage solids that are discharged to landfills, incinerators, or agricultural lands.

2. Materials and methods

2.1. Sample Procurement and Characterization

Six samples of food-grade SiO₂ (fumed, pyrogenic, not labeled as “nano”) were obtained from commercial vendors in the USA and China. All samples presented as white powders were sold specifically as food additives, and were considered as “pristine” SiO₂ because they had not yet been mixed or reacted with food matrices. Fourteen food products including personal care products were purchased in supermarkets in Scottsdale, Arizona (USA) in the fall of 2014 (see Table S1).

Details for sample preparation and analytical methods are provided in Supporting Information (SI), and briefly overviewed here. Hydrodynamic diameter and ζ potential were analyzed by PALS (ZetaPALS, Brookhaven Instruments Corp., NY, USA) in a 10 mM potassium nitrate

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4 158 solution from pH 2.5 to 9.5. Scanning transmission electron microscopy (STEM/TEM, Phillips
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6 159 CM-200, USA) or scanning electron microscopy (SEM) equipped with an electron dispersive X-
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9 160 ray (EDX) microanalysis system (Philips XL30-EDAX, USA) was used to acquire 10 to 15
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11 161 images per sample. SiO₂ or food samples containing SiO₂ were dispersed in nanopure water
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14 162 before transferring small aliquots of supernatant liquid onto the surface of a copper grid
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16 163 (TedPella, Inc., CA, USA) or aluminum SEM stub (see SI for details). The primary particle size
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19 164 (i.e., not agglomerates) in TEM images was analyzed using the Image J software by averaging on
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21 165 250–260 primary particles in each sample (see SI). Food-grade SiO₂ was dried at 60 °C for 24
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23 166 hours before XRD (Siemens D5000, TX, USA) and XPS (ESCALAB 220i-XL, Vacuum
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26 167 Generators, USA) analysis. Elemental composition of samples was determined using inductively
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31 169 microwave digestion in the presence of tetramethylammonium hydroxide (TMAH, 25% in water,
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33 170 J.T. Baker, Avantor Materials, PA, USA).

34 35 36 171 **2.2. Dissolution potential of food-grade SiO₂ in different media**

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41 173 (Yang et al., 2014a). Food-grade SiO₂ at 1000 mg/L was placed in nanopure water, 1 mM
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49 176 DMEM cell medium (Cellgro, 10-013-CM, Mediatech, VA, USA). Liquid media without added
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51 177 SiO₂ were used as controls. Dry powders were added to the liquids in 50 mL plastic centrifuge
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54 178 tubes and dispersed using a vortex mixer (Fisher Scientific, USA) for 1 minute. Separate aliquots
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56 179 (5 mL) were collected for pH and dissolved Si analysis. The remaining solutions were placed on
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59 180 a horizontal shaker for 24 hours, after which samples were collected for dissolved Si analysis.

Dissolved Si was operationally defined as the Si concentration passing through 30 K Da ultracentrifugation membrane filter (cellulose, 30, 000 NMWL, Amicon Ultra, USA) (Yang et al., 2014a). Si was measured by ICP-MS after microwave digestion (see SI). The potential morphology changes of SiO₂ powders were examined by TEM.

2.3. *In vitro* assays: brush border-expressing human cell

The brush border-expressing human cell line, Caco-2 BBe1 (hereafter referred to as Caco-2; CRL-2102) was purchased from American Type Culture Collection (ATCC) at passage number 35 and used before passage number 47 (Peterson and Mooseker, 1992). This cell line was chosen because it was shown to faithfully mimic morphology (Peterson and Mooseker, 1993) and recruit the correct proteins to the brush border *in vitro* at the molecular level. The cell culture medium for use with Caco-2 cells was DMEM (Cellgro, 10-013-CM, Mediatech, VA, USA) supplemented with 10% fetal bovine serum (Atlanta Biological, S10, Norcross, GA, USA), 1% antibiotics (Cellgro, 30-004CI, Mediatech, VA, USA), and 10 µg/mL human transferrin (Invitrogen, 30124SA, Life Technologies, NY, USA). Cells were cultured continuously for 21 days to permit brush border morphogenesis and differentiation of the epithelium (Peterson and Mooseker, 1992). At the end of day 21, cell culture medium containing concentrations of SiO₂ was applied to the epithelia for 24 hours. The time point chosen for exposure in this study is consistent with the current body of literature on the subject (Faust et al., 2014a; Fisichella et al., 2012; Koeneman et al., 2010b; Reed et al., 2014). The sample preparation of epithelia for SEM analysis can be found in SI. Eventually samples were sputter coated with Pt/Au and imaged with either a JOEL JSM3600 SEM or a Phillips XL-30 SEM. For each experiment, >1 mm² of surface area was analyzed from three independent experiments, where averages were generated from 3–5 of 1 µm² static regions of interest in each micrograph. Micrographs were captured at 10,000 x

magnification by choosing 3–5 regions randomly at low magnification where the brush borders could not be seen, and subsequently magnifying to 10,000 x. In order to count microvilli, 3–5 regions ($1\ \mu\text{m}^2$), determined from calibrated scale bars acquired from the electron microscope, were drawn on a transparency. These regions of interest did not change between micrographs in order to avoid potential bias between micrographs. The transparency was overlaid and taped on the computer screen and microvilli were counted by visual inspection of the regions of interest. Microvilli were counted and averaged from 10–11 images acquired at 10,000 x magnification. For control specimens, about 1,500 microvilli were counted corresponding to ~4% of the total surface area imaged. This method for counting microvilli produced results in control specimens that were similar to results reported elsewhere (Peterson and Mooseker, 1993). Images of brush borders originally captured at 10,000 x can be found elsewhere (Faust et al., 2014a; Faust et al., 2014d; Reed et al., 2014). Micrographs were originally captured at 10,000 x for reasons described elsewhere (Faust et al., 2014c).

For detection and analysis of ROS, the human Caco-2BBE1 cell line was grown on 22 mm no. 1 German glass (Deckglaser, 633029, Carolina Biological, NC, USA) for 21 days. At the end of day 21, medium containing 1 mg/L of food-grade SiO_2 (sample 1) was applied to the epithelia for 24 hours. The epithelia were incubated with 10 μM of the ROS indicator H2DCFDA (#D-399, Life Technologies, WI, USA) for 1 hour at 37°C prepared in complete cell culture medium. The epithelia were washed once with calcium/magnesium-containing phosphate buffered saline (PBS), and the samples were immobilized on 10 μL drops of culture medium on glass slides. Three 20 x magnification fields from 3 independent experiments were analyzed (corresponding to a total area of $8.6\text{E}6\ \mu\text{m}^2$) for each experimental condition. For each specimen, phase-contrast

optics were used to focus the specimen, and 3 randomly chosen fields were captured. The number of ROS positive cells within the entire field of view were counted and averaged. Fluorescent images were overlaid with phase-contrast images that clearly delineate boundaries between cells. Boundaries appear as the classic phase-bright “honeycomb” appearance as shown elsewhere (Anderson et al., 1989). Only cells that retained a cell body that overlaid green were scored as ROS positive. Aside from the application of SiO₂, all specimen culture media were treated the same. Averages represent the surface area within the entire 20 x field of view.

2.4. Occurrence and removal potential of food-grade SiO₂ in wastewater treatment

Samples of returned activated sludge (RAS), final sludge (combination of primary settled sludge and wasted activated sludge), influent, and secondary effluent wastewater were collected in fall 2014 from a WWTP in the metro-Phoenix region. The concentration of total Si was determined after TMAH digestion. Cloud point extraction (CPE) can enrich a variety of other nanoparticles in the surfactant phase with a factor of 80 folds (Hartmann et al., 2014;Hartmann et al., 2013;Liu et al., 2009). Colloidal Si was enriched and obtained by CPE on wastewater influent and effluent samples in the summer of 2104, which were collected in 1 L Nalgene bottles. Wastewater samples were filtered with 1.2 µm glass fiber filter, and the filtrates were further prepared following a published CPE method (Hartmann et al., 2014;Hartmann et al., 2013). Briefly, a 40 mL water sample was mixed with 400 µL of 1.25 M sodium acetate solution, 100 µL of 1 M acetic acid, 1.0 mL of saturated EDTA solution, and 1.0 mL of a 10% TX-114 solution (w/w in water). The mixed solution was vortexed in a polypropylene centrifuge tube (VWR, USA), incubated at 40°C in a water bath for 30 min, centrifuged at 2650 G for 12 min, and subsequently cooled at 4°C in the fridge. The aqueous supernatant solution was discarded, and 0.5 mL of the enriched organic phase was further used for TEM/EDX analysis. We validated the procedure of

CPE on gold nanoparticles and nano SiO₂ in the nanopure water matrix and achieved recoveries of 90%–110%.

The removal potential of food-grade SiO₂ by wastewater biomass was determined following previously developed protocols (Kiser et al., 2010) using sludge from a lab-scale sequencing batch reactor that was maintained for 2 years without any exposure to nanomaterials. The sludge from the lab-scale reactor exhibits similar nanomaterial removal characteristics as RAS from full-scale WWTPs (Kiser et al., 2010; Wang et al., 2012; Yang et al., 2014b) and alleviated concern regarding contamination from SiO₂ particles already present in full-scale RAS. A matrix of experiments was conducted in 50 mL plastic centrifuge tubes by varying the amount of well-dispersed food-grade nanoSiO₂ (0 or 1 mg Si/L) and biomass concentration (0 or 1000 mg total suspended solids [TSS]/L) in 1 mM NaHCO₃. Samples were mixed for 3 hours and allowed to settle for 30 minutes before the supernatant was sampled for total Si concentration by ICP-MS after TMAH digestion.

2.5. Statistical analysis

Statistical analysis was conducted by the software SPSS 11.5 for Windows (SPSS Inc., Chicago, IL, USA). The mean of microvilli numbers among the control and six samples of food-grade SiO₂ were analyzed by one-way ANOVA using Tukey's post-test. The ROS values were also compared using one-way ANOVA for the negative control, the microvilli dosed with 1 mg/L SiO₂, and the positive control. The normality of microvilli numbers for each treatment was examined using Shapiro Wilks W Test. The homogeneity of variances of microvilli number for

each treatment was tested before ANOVA analysis by Lavene's test. The level of significance was set as $p < 0.05$ for all the tests.

3. Results and discussion

3.1. Primary particle size and hydrodynamic diameter of food-grade SiO₂

Bright field TEM images for six "pristine" bulk food-grade SiO₂ samples are shown in Fig. 1. All samples contained agglomerates of SiO₂ particles, and none showed evidence of any crystal structure on the particles under high-resolution mode of TEM (Images are not shown). Fig. S1 shows the number-based particle size distribution. The mean diameters of all primary SiO₂ particles were < 100 nm, with mean primary particle sizes in a narrow range of 9 to 26 nm. Specifically for the primary particles in samples 1- 6 ($n = 250$ for samples 1-5, and $n = 260$ for sample 6), mean diameters ($\pm$ one standard deviation [SD]) were 22 ± 8 , 26 ± 17 , 15 ± 10 , 19 ± 5 , 17 ± 9 , and 9 ± 6 nm, respectively. Thus, food-grade SiO₂ mentioned hereafter in this paper is considered nano SiO₂. While the primary particles measured by TEM were < 100 nm, agglomerate sizes were several hundred nanometers. The hydrodynamic diameters (i.e., effective diameter in PALS measurement, five replicate measurements) of agglomerates in samples 1–6 were 1579 ± 88 , 1223 ± 468 , 1477 ± 204 , 1759 ± 265 , 1151 ± 108 , and 1539 ± 146 nm, respectively. These values were much larger than the primary particle diameters measured by TEM, indicating the occurrence of SiO₂ agglomerates when suspended in water.

3.2. Crystal structure and surface functionality of food-grade SiO₂

The XRD spectra of samples 1–6 (Fig. S2) exhibited a broad peak with a 2θ value of 21.6° , which differed from the ground quartz powder (gives $2\theta = 26.5^\circ$ and $d = 0.336$ nm) (Athinarayanan et al., 2014; Hess, 1995). This indicates markedly disordered atom arrangements of the [SiO₄] tetrahedron units in SiO₂ (Hess, 1995), signifying that all of samples tested were

amorphous SiO₂. Fig. 2 also shows the wide-scan and Si 2p XPS spectra for sample 1, which is representative of all the samples (shown in Fig. S3). The O 1s spectra showed a peak at 525.7 eV and Si 2p spectra had a peak at 103.7 eV. The National Institute of Standards and Technology (NIST) database shows the binding energy ranges of Si at different valences (Naumkin et al., 2012), which are 98.7 – 99.6 eV for Si⁰, 101.7 – 102 eV for Si²⁺, 101.7 – 103.8 for Si³⁺, and 103.0 – 104.1 eV for Si⁴⁺. Closer observation on Si 2p spectra (Fig. 2) with a binding energy of 103.7 eV revealed the Si 2p peak was composed of two separate peaks upon the peak fitting results, which indicated the majority of silicon presented as Si (IV) with a small amount of silicon (III) (Wang et al., 2011). The incomplete oxidation state of SiO₂ surface was likely due to the non-equilibrium combustion during the production process, which involves the hydrolysis of silicon tetrachloride in a hydrogen/oxygen flame (Bergna and Roberts, 2005; Wang et al., 2011). Modeling by density functional theory indicates that a silicon (III) site on the surface of SiO₂ can be restructured to generate a formal Si (IV) site when binding with water molecules (Wang et al., 2011), accompanied by a potential generation of hydroxyl (OH·) radical during the oxidation/reduction process (Zhang et al., 2012).

3.3. Zeta (ζ) potential and elemental composition of food-grade SiO₂

Fig. 3 shows ζ potential measurements of food-grade SiO₂ in a 10 mM potassium nitrate solution from pH 2.5 to 9.5. The isoelectric point of all samples was below 3.2. In the typical pH range for natural and tap waters (i.e., pH 6 – 8), the ζ potential values of all samples ranged from -5 mV to -20 mV. Sample 2 exhibited the largest ζ potential range, from +5 to -34 mV, while the other samples exhibited less pH dependence. Sample 2 was the only sample without detectable levels of metals (Na, Al, Ca, or Fe—see Table S1). Elemental composition (Table S1) showed the major element in samples 1–6 was silicon (39–49% w/w), with a few minor elements less

than 1% (w/w) such as sodium (Na, 0.3 – 0.58% w/w), aluminum (Al, 0.02 – 0.3% w/w), and calcium (Ca, 0.02 – 0.04% w/w); oxygen was assumed to be the other element present. Sample 4 contained the highest levels of Na and Al, had the most negative ζ potential, and showed a ζ potential profile with the least pH dependence. While the concentrations of these metals were low ($< 0.6\%$), it appears that increased metal content often decreased the measured ζ potentials when $\text{pH} < 7$, which might be due to the generation of aqueous aluminum (hydr-)oxides when aluminum oxide in SiO_2 powder reacts with protons in the water (Kravchenko et al., 2005). Due to the presence of multiple other metallic elements in SiO_2 powder, further research is needed for the exact mechanism of ζ potential change.

3.4. Morphology and concentrations of SiO_2 in real food samples

Fig. 4 presents representative parallel SEM and TEM images of SiO_2 extracted from foods. SEM images, shown in secondary electron mode, show large agglomerates of particles containing silicon, with 1.3 – 7.5 μm agglomerate sizes. However, the carbon coating on the SEM sample stub surface and resolution limit of SEM (i.e., 10 – 15 nm on XL 30 under this working conditions) made it difficult to confirm the presence of nanosized SiO_2 (9 – 26 nm). The SEM coupled EDX shows the morphology of large agglomerates and presence the silicon in foods. Higher resolution TEM images clearly revealed the agglomerates of nano-sized SiO_2 , which were 22 ± 4 , 25 ± 17 , and 18 ± 3 nm of the diameters of primary particles in samples 1, 3, and 4, respectively. Ten of fourteen different foods contained primary particles that were < 100 nm in size (TableS1). EDX spectra confirmed that the main elements were silicon and oxygen (copper detected was from copper grid). No crystal structure was identified under HRTEM mode, which was consistent with the XRD data and supported the interpretation that SiO_2 present in foods and personal care products had amorphous structure (Lim et al., 2015). As the mass detection limit

by EDX is about 0.5% (by weight), the SEM/EDX or TEM/EDX analysis can provide the composition of main elements. However, the precise element composition analysis can only be conducted after digestion and following ICP-MS analysis, especially for those elements with concentration lower than 0.5% by weight.

The silicon concentrations in 14 foods and consumer goods ranged from 1.3 to 16.3 mg Si/g dry foods (i.e., 0.13% – 1.63% by weight) except for one toothpaste sample (Table S1). Therefore, most food samples contained less than the 2% weight percentage set in the code of USFDA (USFDA, 2014). The toothpaste sample contained the highest silicon concentration of 189.0 ± 3.9 mg Si/g dry mass, which was most likely from SiO₂ gel and or silicate salts in toothpaste because no SiO₂ nanoparticles were observed in this sample (ADA, 2014). If the silicon concentration is normalized per suggested serving size, the silicon contents per serving range from 2.8 mg Si/serving (Vitamin D3 Tablet) to 199 mg Si/serving (Lemon Supreme Cake Mix).

To assess representative exposures in the GI tract to SiO₂ in food, we assumed serving sizes and total area of the small intestines with microvilli. In context of daily SiO₂ intake, two serving sizes of cake mix (i.e., 199 mg Si/serving) can lead to an intestine exposure rate of 160 ng Si/cm² (considering a total small intestine area of 250 m²) (UPMC, 1999). When being converted to laboratory scale conditions, this exposure rate equates to a concentration of 0.56 mg Si/L culture media (i.e., 1.2 mg SiO₂/L) according to the ratio (100 ng nanomaterial/cm² microvilli equals to 0.35 mg nanomaterial/L culture media) (Faust et al., 2014a). Thus, the concentration of 1 mg

SiO₂/L used in the microvilli study represents a realistic exposure concentration, which is also suitable for *in vitro* assays. These values are used in subsequent sections.

3.5. Dissolution of food-grade SiO₂ in different media

Fig. 5 shows dissolved silicon concentration at initial conditions ($t = 0$) and after a 24 h dissolution potential test in Nanopure water, 1 mM NaHCO₃, and tap water (initial concentration of 1,000 mg SiO₂/L). After 24 hours, the food-grade SiO₂ released 1.3–4.9 mg Si/L (i.e., 0.3–1.1% normalized to 1,000 mg SiO₂/L) and 4.2–16 mg Si/L for Nanopure water and 1 mM of NaHCO₃ solution. The obtained values of dissolute SiO₂ ranged from 17 to 32 mg Si/L in tap water after 24 hours and were higher than the ones from other water matrices. The equilibrium pH after dosing SiO₂ in water matrix of sodium bicarbonate, tap water, and Nanopure water were 8.4 ± 0.2 , 8.4 ± 0.0 , and 7.0 ± 0.7 , respectively. This suggests that higher pH along with higher ionic strength (i.e., conductivity) could facilitate dissolution of food-grade SiO₂, especially in context of higher conductivity of the sodium bicarbonate (72 μ S/cm) and tap water (400 μ S/cm) than Nanopure water (0.055 μ S/cm [equal to 18.2 m Ω .cm]), which is consistent with other reports for amorphous SiO₂ (Icenhower and Dove, 2000). Although these SiO₂ concentrations indication a dissolution potential for food-grade SiO₂, they are significantly lower than the saturation concentration of silicon at room temperature in this pH range, which is between 70 and 80 mg Si/L (Richard Drees et al., 1989). Nonetheless, this work shows the *potential* for amorphous SiO₂ to dissolve in various water matrices, and dissolved SiO₂ could reach 6.8% of the applied amorphous SiO₂.

3.6. Food-grade SiO₂ nanoparticles affect on brush border microvilli

As an indicator of the potential biological effects along the supply chain of SiO₂ nanoparticles, the effect on microvilli in the digestive tract was studied. Previously, we observed adverse effects from a range of nanoparticles on microvilli (Faust et al., 2014b; Koeneman et al., 2010a; Zhang et al., 2011). Brush border disruption was originally defined as a net loss and change in the normal arrangement of brush border microvilli within the *in vitro* epithelium (Koeneman et al., 2010b). This non-lethal, albeit critical, effect of exposure to additives could result in decreases in absorption of nutrients because microvilli exist at the apex of the cell in order to increase the total surface area of the intestine. In this study, we employed the archetypical organization of the brush border in order to identify whether or not food-grade nano SiO₂ disrupted the normal organization of brush border microvilli. Under normal conditions, and when viewed with the SEM, brush border microvilli appear as thin, finger-like projections emanating from the cell apex (Faust et al., 2014a; Hirokawa et al., 1982; Mooseker, 1985; Tilney and Mooseker, 1971). Fig. 6A shows one such untreated control. At this magnification (originally captured at 10,000 x), a large number of microvilli exist, making it impossible to see the underlying plasma membrane. Consistent with a number of other reports (Peterson et al., 1993; Peterson and Mooseker, 1993), the present investigation found 42 ± 8 microvilli/ μm^2 (MV/ μm^2 ; mean $\pm$ SD) on the apical surface of untreated control cells (Fig. 6H). However, exposure to 6 different food-grade SiO₂ additives resulted in a net loss and disorganization of brush border microvilli in replicate samples at a concentration of 1 mg/L (Fig. 6B–5G). For sample1, the organization of microvilli appeared to fall over on their sides (Fig. 6B). Analysis of the total number of microvilli on the surface indicated a 43% loss of microvilli to a value of 24 ± 8 MV/ μm^2 compared to untreated control specimens (Fig. 6H). Exposure to samples 2–6 resulted in microvilli falling on sides (Fig. 6C–5G), and 23 ± 8 , 25 ± 7 , 27 ± 5 , 26 ± 6 , and 23 ± 8 MV/

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4 405 μm^2 , respectively (Fig. 6H, n = 9 from 3 independent experiments). For each of the specimens,
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6 406 EDX analysis coupled with morphological views of the material indicated that the surfaces of
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9 407 exposed epithelia were SiO_2 positive (Fig. S5 and S8). Dosimetry analysis of sample 1 suggested
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11 408 that concentrations as low as 0.01 mg/L of SiO_2 elicit significantly ($p < 0.01$) fewer *in vitro*
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13 409 brush border microvilli compared to untreated controls (Fig. S6). Sample 1 was an appropriate
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15 410 representative of all samples in this evaluation for the dosimetry test because there was a similar
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18 411 magnitude of brush border disruption among all six samples (Fig. 6).
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25 413 Recent studies showed that exposure to food-grade TiO_2 additives (Faust et al., 2014a) and drink
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27 414 supplements that contained nanoparticles resulted in disruption of brush border microvilli *in vitro*
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29 415 (Reed et al., 2014). From the typical modes of toxicity of nanomaterials on microvilli or other
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31 416 cell lines (Faust et al., 2014d; Zhang et al., 2010), the impact of food-grade SiO_2 on microvilli cell
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33 417 lines could be partially the result from the dissolution of SiO_2 and/or the generation of ROS
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35 418 (Steven, 2011; Yang et al., 2013). Data presented in this manuscript indicate that there was no
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37 419 leaching of silicon ions into the culture media from the nanomaterial at 1 mg/L of SiO_2 (Fig. S4).
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39 420 However, the generation of ROS is expected because the presence of Si (III) on the surface
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41 421 confirmed by XPS (Fig. S3) could lead to hemolytic cleavage of the Si-O bond on the SiO_2
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43 422 surface and generate a $-\text{Si}-\text{O}\cdot$ on the surface. The $-\text{Si}-\text{O}\cdot$ would further react with water
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45 423 molecules to generate $\text{HO}\cdot$ radicals (Zhang et al., 2012).
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56 425 Generation of $\text{HO}\cdot$ radicals as oxidative stress could alter the normal physiology of the cells
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58 426 within the epithelium that give rise to well-defined brush borders. Indeed, studies have shown
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that application of Fe₂O₃ nanoparticles disrupt intercellular junctions (Kalive et al., 2012;Zhang et al., 2010). Although the mechanism of disruption of junctions as a consequence of nanoparticle exposure remains to be elucidated, studies suggest that increases in oxidative stress result in disruption of intercellular junctions (Bailey et al., 2004;Rao, 2008;Sheth et al., 2003). It is known that proteins responsible for the establishment of the terminal web are intimately connected to adherens junctions within the gut epithelium (Hirokawa and Heuser, 1981;Hirokawa et al., 1982). Because the terminal web is necessary for elongation of microvilli, it might be expected that changes in intercellular junctions could, in part, result in retraction/limp brush border microvilli. For these reasons, it was necessary to confirm if exposure to the nanomaterial resulted in changes in the level of intracellular ROS.

The ROS indicator H₂DCFDA has been used in this study to determine changes in ROS within living cells in culture (Böhmer et al., 1992;Chompoosor et al., 2010;Faust et al., 2014a;Li et al., 2012). Under normal conditions, untreated control Caco-2BBE1 epithelia had 7 ± 6 (mean $\pm$ SD, n = 9 from 3 independent experiments) ROS positive cells (Fig. S7) within the 20 x field of view (see details in materials and methods). However, exposure to 1 mg SiO₂/L resulted in a statistically significant increase (p < 0.001, n = 9 from 3 independent experiments) to 28 ± 9 ROS positive cells within the epithelium (Fig. S7). It was important to assess the relative magnitude of ROS increased between untreated controls and epithelia exposed to SiO₂. In order to do this, the phorbol ester, phorbol-12-myristate-13-acetate (i.e., PMA, TPA, etc.) was used to elicit high levels of ROS (Eruslanov and Kusmartsev, 2010). PMA exposure as a positive control for ROS resulted in 50 ± 17 ROS positive cells within the field (Fig. S7, n = 9 from 3 independent experiments). These data indicate that exposure to food-grade SiO₂ resulted in

increased production of intracellular ROS. Because ROS production is known to be propagated (Thubagere and Reinhard, 2010) and affects junction integrity (Araki et al., 2005; Banan et al., 2004; Schreibelt et al., 2007), and because adhering junctions are noted to be intimately connected with the terminal web that gives rise to brush border microvilli (Bement and Mooseker, 1996), changes in junction integrity through the production of ROS could be one mechanism responsible for the reduction in the number of brush border microvilli observed as a consequence of exposure to SiO₂.

3.7. Occurrence and removal potential of food-grade SiO₂ in wastewater treatment

The use/disposal of foods and or food-grade SiO₂ results in their release to sewer system and eventually at the influent to WWTP. Thus, CPE (i.e., cloud point extraction) coupled with TEM/EDX analysis was conducted to explore the presence of food-grade SiO₂. Fig. 7a shows the presence of amorphous SiO₂ agglomerates, which exhibit an average primary particle diameter of 20.7 ± 3.8 nm in the CPE phase extracted from raw sewage. The agglomerate showed a similar primary particle size and structure to food-grade SiO₂, indicating the potential release of food-grade SiO₂ to sewage. We did not find any agglomerates of amorphous SiO₂ in the effluent wastewater. However, we identified a few individual SiO₂ particles with an average diameter of 21.5 ± 4.7 nm in treated effluent from the WWTP. While these are the same size as the SiO₂ primary nanoparticles in food, they could be from other sources (e.g., polishing agents – Speed et al., 2015) or de-agglomerated SiO₂ from food materials.

The measured SiO₂ concentration in the enriched cloud phase (surfactant phase containing nanomaterials – Figure 6) was 0.6% and 0.5% for the raw sewage and treated effluent samples,

respectively. The silicon concentrations (filtered through 1.2 μm filter) in raw sewage, secondary effluent of a local WWTP were 88.6 ± 2.6 and 116 ± 15 mg Si/L, respectively, which were close to the equilibrium solubility of amorphous SiO_2 in water at $\text{pH} < 8.5$ (Morey et al., 1964). A number of factors could contribute to higher filtered SiO_2 concentrations in the WWTP effluent when compared to influent concentrations. First, there is roughly a lag time of 6 to 10 hours resulting from the hydraulic residence time in a WWTP. Second, the agglomerates of amorphous SiO_2 identified in the influent of WWTP were not found in the effluent, while discrete nano-scale SiO_2 were found in the effluent of WWTP. This indicated that larger particles or their agglomerates ($d_p \geq 1.2 \mu\text{m}$) may have sheared to form smaller particles that passed through our filtration step. Although present, these discrete nanoparticles could be considered an irregular occurrence rather than dominant one because majority of the SiO_2 has a tendency to accumulate in the biosolids. We measured the silicon concentration in the RAS and final biosolids, which were 3.72 ± 0.26 and 6.75 ± 0.19 mg/g dry biosolid, respectively, indicating a possible trend of increased silicon concentration in biosolids after combining primary settling sludge and wasted activated sludge (the same composition as returned activated sludge).

This tendency for SiO_2 to associate with wastewater biomass was further supported by our laboratory batch tests where we screened for the potential distribution of food-grade SiO_2 to associate with wastewater biomass (i.e., solid-liquid distribution ratios). For six samples of food-grade SiO_2 , $70 \pm 1\%$ of SiO_2 was removed from the liquid phase in the presence of 1 g TSS/L biomass. As shown in Fig. 7a, the agglomerates of SiO_2 can sorb on the sludge and thus precipitate in the settling stage. Because a typical WWTP usually has a higher activated sludge concentration (2–4 g/L) and higher ionic strength, a higher removal rate of food-grade SiO_2 is anticipated in a WWTP.

Conclusions and Implications

Overall, the six bulk food-grade SiO₂ (E551 designation) samples evaluated in this study were similar, containing both Si (III) and Si (IV) surface oxidation states and amorphous SiO₂ with primary particle sizes (9 to 26 nm) within larger agglomerates of 0.5 to 2 µm. Actual food samples contained SiO₂ (1.3–16.2 mg Si/g food) and had similar morphology and sizes as the bulk E551 samples. The uptake of these foods or use of personal care products containing food-grade SiO₂ could lead to dermal and oral exposure of nano SiO₂ to humans. Previous acute toxicity studies on amorphous food-grade SiO₂ demonstrated it does not show any lethal effects during inhalation exposure of rats at the highest concentration of 2 mg SiO₂/L (OECD, 2004). However, we observed the disruption of structure of food-grade SiO₂ on microvilli at 1 mg SiO₂/L, a dose consistent with exposure during ingestion of foods, during *in vitro* testing and the production of ROS. Another *in vitro* intestinal brush border microvilli assays showed a consistent inhibitory pattern where food-grade SiO₂ associated with microvilli and caused microvilli disruption. Loss of microvilli could impact nutrient uptake in the gastrointestinal tract. As the entire mucosal layer including microvilli is turned over every 4–5 days in mammals, the impact of food-grade SiO₂ on human microvilli would be minimal, though effects of continuous uptake of foods containing food-grade SiO₂ still needs to be further explored.

To better understand the fate/change of food-grade SiO₂ through its lifetime, we performed three “functional assays” to screen for (1) dissolution potential, (2) ability to disrupt important physiological processes, and (3) ability to distribute between wastewater biomass solid to liquid phases. Functional assays are experiments intended to quantify the next effects of intrinsic

nanoparticle properties (size, degree of agglomeration, zeta potential, surface functionality, crystal structure, etc.). First, our dissolution potential tests showed that higher pH and ionic strength increased SiO₂ dissolution, which is consistent with more detailed studies (Hess, 1995; Icenhower and Dove, 2000). Second, a number of studies have shown nanoparticles, including nanoparticles from consumer goods and food additives, disrupt brush border microvilli (Faust et al., 2014a; Koeneman et al., 2010b; Reed et al., 2014). Our *in vitro* assay showed that food-grade SiO₂ dosed at levels close to predicted exposure levels (< 1 mg Si/L or as low as 10 ng/cm² of epithelium) disrupted brush border. Third, our wastewater biomass test showed the potential for > 70% removal of food-grade SiO₂ during biological treatment using activated sludge processes. We suggest greater than 70% because the screening test was performed with 1g TSS/L, which is lower than the typical biomass concentrations of 2 to 4 g TSS/L. The emergence of functional assays for engineered nanomaterials may provide screening-level assessments of where additional, more detailed, studies are appropriate to understand the fate of engineered nanomaterials (Hendren et al., 2013; Westerhoff and Nowack, 2013). Currently there is still a large uncertainty on the characteristics and impacts of engineered nanomaterials on environment and humans. A suite of standardized functional assays that can provide a basis to compare and justify the effects of nanomaterials will be useful if developed. For example, dissolution study and biomass removal rate test can be potentially developed to standard protocols as a basis for assessing engineered nanomaterial transformation and fate, though future research and collaboration is needed for it. Assays employed here provide an improved understanding and an example of the characterization, transformation, and potential effects of food-grade SiO₂ across its value chain in food products. This work provides the first detailed

study of food-grade SiO₂ across its life cycle, which is a useful model for approaching the transformations and impacts of other, potentially even more reactive, engineered nanomaterials.

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Fig. 1. Bright field TEM images of raw food-grade silicon dioxide powders (size distribution is presented in Fig. S1).

Fig. 2. X-ray photoelectron spectroscopy on food-grade silicon dioxide powders (Sample 1, wide-scan and Si 2p spectrum; analysis on other samples with similar spectrum are shown in Fig. S3).

Fig. 3. ζ potential of food-grade silicon dioxide suspensions over a range of pH levels. The 7 replicate measurements were conducted for each ζ potential value.

Fig. 4. Top three images are SEM images of food samples (a, food #1; b, food #3; c, food #4), and the bottom three images are bright field TEM images of the same source of food samples after extraction from water (d, food #1; e, food #3; f, food #4). Major elements detected by energy x-ray dispersive spectroscopy (EDX, Fig. S9) are listed at the bottom of each image. The scale bar represents 500 nm, 2 μm, and 500 nm in images a, b, and c, respectively and represents 100 nm in images d, e, and f.

Fig. 5. Dissolution potential test results for food-grade silica in Nanopure water (a), 1 mM NaHCO₃ solution (b), and tap water (c) showing dissolved Si concentration after ultrafiltration. The initial solid phase dose was 1,000 mg SiO₂/L in each test. C1, C2, and C3 were the negative controls (i.e., no silica) with only nanopure water, 1 mM NaHCO₃ solution, and tap water, respectively. Error bar represent data range of duplicate test, and each test was measured in triplicate.

Fig. 6. SEM images indicated disruption of the brush border as a consequence of SiO₂ nanoparticle exposure. Images represent untreated intestinal brush borders (A), intestinal brush borders exposed to silica sample1 (B), sample 2 (C), sample 3 (D), sample 4 (E), sample 5 (F), and sample 6 (G). Fig. H (the histogram) demonstrates the relative change between epithelia exposed to food grade SiO₂ nanomaterial. The corresponded EDX spectrum is shown in Fig. S10. Comparisons were made between untreated brush borders and experimental samples alone. In each micrograph the scale bar shown in the lower right corner is 2 μm, and the white arrows point to the putatively agglomerated SiO₂ nanomaterial. The histogram represents averages from 3 independent experiments where n = 9. The images shown were originally captured at 10,000 x.

Fig. 7. TEM image of amorphous SiO₂ aggregates in raw sewage entering into WWTP (a) and nano silica particles in treated WWTP effluent (b). Samples were enriched by cloud point extraction from the water samples, and then characterized by HRTEM. The red boxes in images were spots where EDX was conducted. The corresponded EDX spectrum is shown in Fig. S11.