# Presence of Nano-Sized Silica during *In Vitro* Digestion of Foods Containing Silica as a Food Additive

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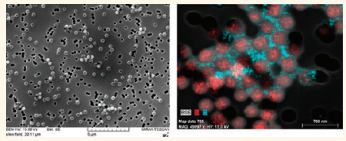
**ABSTRACT** The presence, dissolution, agglomeration state, and release of materials in the nano-size range from food containing engineered nanoparticles during human digestion is a key question for the safety assessment of these materials. We used an *in vitro* model to mimic the human digestion. Food products subjected to *in vitro* digestion included (i) hot water, (ii) coffee with powdered creamer, (iii) instant soup, and (iv) pancake which either contained silica as the food additive E551, or to which a form of synthetic amorphous silica or 32 nm $SiO_2$ particles were added. The results showed that, in the mouth stage of the digestion, nano-sized silica particles with a size range of 5−50 and 50−500 nm were present in food products containing E551 or added synthetic amorphous silica. However, during the successive gastric digestion stage, this nano-sized silica was no longer present for the food matrices coffee and instant soup, while low amounts were found for pancakes. Additional experiments showed that the absence of nano-sized silica in the gastric stage can be contributed to an effect of low pH combined with high electrolyte concentrations in the gastric digestion stage. Large silica agglomerates are formed under these conditions as determined by DLS and SEM experiments and explained theoretically by the extended DLVO theory. Importantly, in the subsequent intestinal digestion stage, the nano-sized silica particles reappeared again, even in amounts higher than in the saliva (mouth) digestion stage. These findings suggest that, upon consumption of foods containing E551, the gut epithelium is most likely exposed to nano-sized silica.



**KEYWORDS:** *in vitro* digestion · risk assessment · silica · nanoparticles · food · intestine · fate

Synthetic amorphous silica (SAS), often referred to as silica ($SiO_2$), is widely applied in food products and registered within the EU as a food additive (E551). It is mainly used to thicken pastes, as an anticaking agent to maintain flow properties in powdered products, and as a carrier for fragrances or flavors in food and nonfood products.[1,2] While the European regulations allow different production methods for SAS in E551,[3] a prominent production method of SAS is by means of flame hydrolysis from $SiCl_4$ according to the so-called "Aerosil" method.[4] $SiCl_4$ is burned in a hydrogen flame at temperatures ranging from 1000 to 2500 °C, resulting in the production of primary silica nanoparticles. According to the most recently established criteria, SAS is a nanostructured material.[5−7] The primary particles with sizes of approximately 10 nm aggregate into particles with sizes on the order of 100 nm, which agglomerate in turn to particles in the larger nano- or micro-size range[1,4,8,9] (see Figure 1).

Recent research showed that in powdered food materials containing E551 at least part of the silica is in the nano-size range.[10] In general, inorganic silicon compounds may occur as natural components in foodstuffs.[11] Naturally present inorganic silicon compounds occur mostly as sodium, calcium, and magnesium silicates and as hydrated silica, $SiO_2 \cdot nH_2O$. The latter may form small particles in the size range of 1−5 nm and can be found in natural waters, including drinking and mineral waters.[8,12]

The safety of SAS has been evaluated in the past, for example, by the Expert group on Vitamins and Minerals, who set a Safe Upper Level for daily consumption of silica at 700 mg of silica per day for adults.[13] However, SAS used in the studies underlying this evaluation does not meet currently established material characterization criteria.[14] We have shown that up to 33 mass % of silica present in E551 (or SAS) has an external size between 10 and 200 nm.[10] In addition, it is likely that this silica originates from SAS, but the form of SAS used is unknown. The consequences for the

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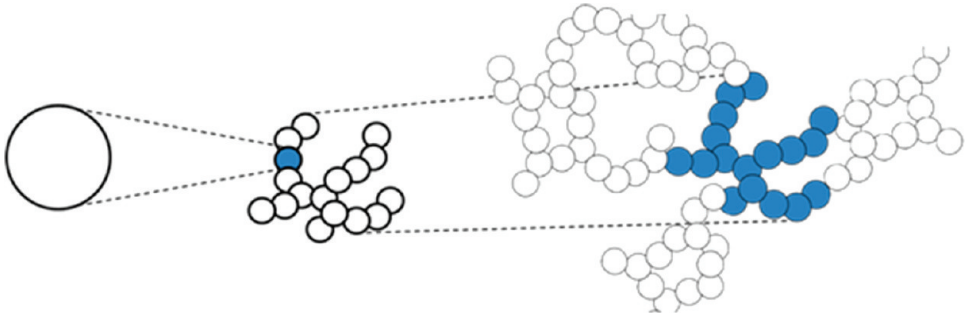

**Figure 1. Sequence of structure development from primary particles into aggregates and finally agglomerates of pyrogenic synthetic amorphous silicas. An aggregate refers to "a group of particles held together by strong forces such as those associated with covalent or metallic bonds", while an agglomerate refers to "a group of particles held together by weak forces such as van der Waals forces, some electrostatic forces and surface tensions".[5]**

potential risks of this nano-sized fraction of the silica materials are uncertain and need to be explored further.[10,15−17] This exploration starts with a notion on the potential for uptake of the nanostructured silica as present in food. Little is known on the gastrointestinal absorption of nanostructured SAS. For example, it is not known whether nanostructured SAS is absorbed in a size-independent manner or whether only the nano-sized fraction can be taken up or taken up at all. Clearly, uptake following ingestion of silica particles cannot be excluded given the reported oral toxicity.[1,9,10,18]

In the absence of data on gastrointestinal uptake and oral toxicity, *in vitro* digestion may be used to investigate the fate of the nanomaterials during digestion and direct future toxicological studies. If SAS dissolves or disintegrates, limited or no further toxicological testing might be needed to investigate nano-size related effects.[19] The stability of silica nanoparticle dispersions has been widely studied in colloid science because of their many applications in soil science, electrochemistry, and polymers. The Derjaguin−Landau−Verwey−Overbeek (DLVO) theory is often used to explain the aggregation/agglomeration behavior of silica suspensions.[20,21] Briefly, the DLVO theory combines the effects of the attractive van der Waals forces and the electrostatic repulsion between charged surfaces. The presence of silanol groups (Si−OH) on amorphous silica surfaces and its charge determine the extent of repulsive energy to keep the silica nanoparticles in dispersion.[22,23] The surface charge of silica nanoparticles becomes different upon changes in pH due to protonation and deprotonation of the silanol groups. At pH 2−3, silica nanoparticles reach the isoelectric point (IEP) where the particles carry no net charge, causing the nanoparticles to aggregate at these pH values.[24] In the case of biological conditions with high (0.1−0.3 M) salt concentrations,[25] ion-specific effects also play a prominent role, and the between-particle force can become attractive at both small (<0.3 nm) and large (4−8 nm) particle distance while repulsive at intermediate distances. This is similar

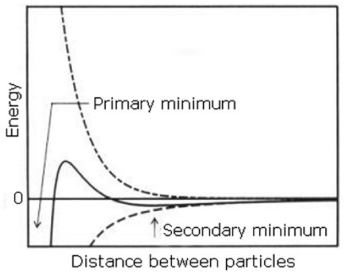


**Figure 2. Schematic diagram of the variation of free energy with silica particle separation at high salt concentrations. DLVO theory predicts the possibility of the existence of a secondary minimum, meaning that silica particle aggregation can be reversible (adapted from ref 25).**

to the secondary minimum found in ordinary DLVO theory (see Figure 2), and as a consequence, particle aggregation can be reversible. All of this is relevant because during human digestion all conditions known to affect aggregation behavior of silica nanoparticles change; for example, pH drops to around pH 2 from mouth to stomach and increases again in the gut to pH 6.5 in the presence of high electrolyte concentrations.

Several models are available to study the fate of compounds during *in vitro* digestion.[19,26] In this study, we used a well-established model that was previously used to study the release of contaminants from soil, food, and toys.[26−29] Briefly, the gastrointestinal tract is simulated for the mouth, stomach, and small intestine using physiological transit times and relevant enzymes. The large intestine is not taken into account because *in vivo* food digestion and absorption mainly take place in the small intestine. This model has not yet been applied for nanomaterials.

In this paper, we studied the presence of nano-sized silica following *in vitro* digestion of foods without and with E551, or with added SAS, or added 32 nm $SiO_2$ particles. We used three model food matrices: hot coffee, instant soup, and pancake. To determine the nano-sized fraction of SAS during the different *in vitro* digestion stages, samples were collected and analyzed using hydrodynamic chromatography coupled to inductively coupled plasma mass spectrometry

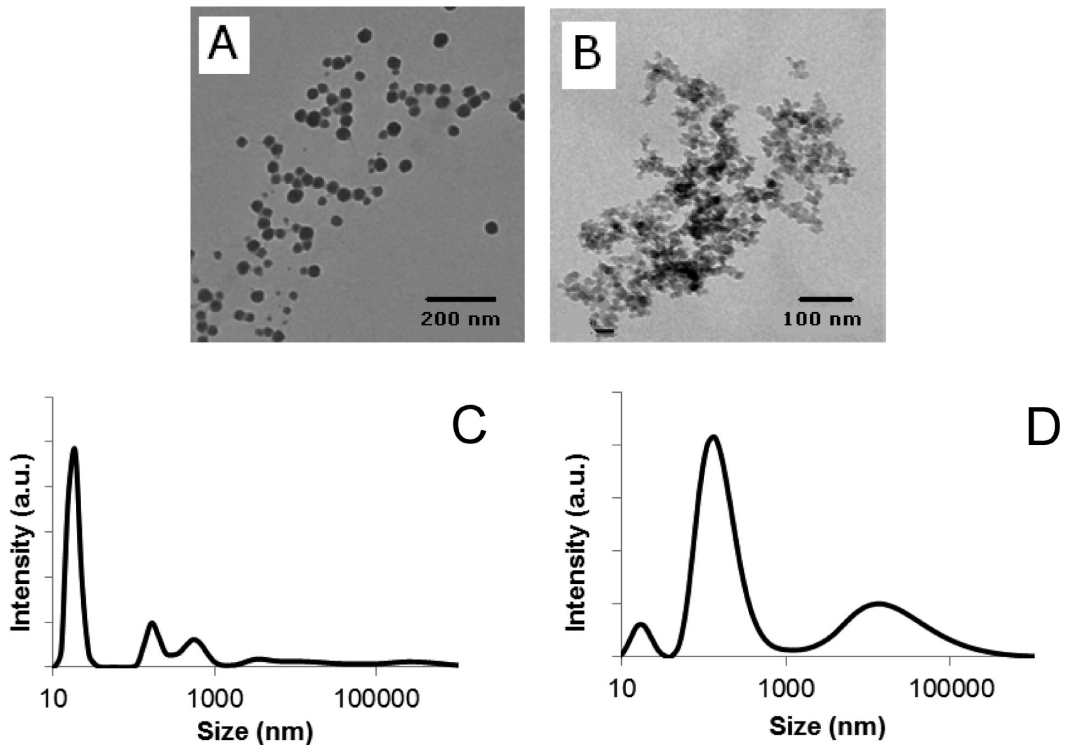


**Figure 3. Characterization of silica nanoparticles used for *in vitro* digestion. TEM images of 32 nm $SiO_2$ (A) and SAS (B). The different gray levels in image B are not due to diffraction contrast, but rather to local stacking of particles. The hydrodynamic size distribution determined by DLS of 32 nm Si-NP (C) and SAS (D) that both were dispersed in artificial saliva (10 $\mu$g/mL) at pH 6.5−6.8 for 2 h (SAS supplemented with 0.05% BSA explaining the additional peak around 20 nm).**

(HDC-ICP-MS) while additional measurements were performed using dynamic light scattering (DLS) and scanning electron microscopy (SEM). Implications of the presence of nano-sized silica for uptake of SAS from food and feed are discussed.

## RESULTS AND DISCUSSION

**Material Characteristics.** The surface area of the SAS used in this study was 388 ± 2.5 $m^2$/g as was determined previously.[10] The primary particle size was 7 nm, while most of the primary particles form larger aggregates and/or agglomerates.[10] As shown in Figure 3, SAS is polydisperse in artificial saliva, with the hydrodynamic size of the main peak determined with DLS around 100 nm. The 32 nm $SiO_2$ particle originates from an interlaboratory comparison for which IRMM (Institute for Reference Materials and Measurements, European Commission Joint Research Centre, Geel, Belgium) prepared a test material TS-2009/1. The results of the interlaboratory exercise showed a mean particle diameter of 31.7 ± 4.1 nm in EM analysis and 35.7 ± 4.1 nm in DLS analysis.[30]

***In Vitro* Digestion of Food Products with E551, Added SAS, or Added *n*-$SiO_2$.** Three different food matrices were tested with or without E551, added SAS, or added *n*-$SiO_2$ (32 nm IRMM test material). All measurements were carried out in duplicate, and the complete experiment was repeated a second time. Water as a matrix and digestion stage was included only as single measurements in the second series of experiments. The results of the measurements summarized in Table 1 are expressed as the mass percentage of the added silica in the size range of 5−200 nm. To extract additional information on the presence of nano-sized silica in each digestion step of the *in vitro* experiment, a one-way blocked analysis of variance (ANOVA) was carried out. In products that did not contain E551, no or only low levels of silica were found during the subsequent stages of the digestion. These levels were comparable with those in blank control samples.

The mean mass percentages reported in Table 1 range from 5% in the digestion of pancake up to 104% for *n*-$SiO_2$ when water was used as a food matrix. The mass percentage of *n*-$SiO_2$ at the subsequent digestion stages reflects the recovery of *n*-$SiO_2$. While a percentage over 100% is not expected, it can occur due to the uncertainty in the HDC-ICP-MS analysis.

The reproducibility of the HDC-ICP-MS analysis can be calculated using the results of the calibration standards that were analyzed between sample series over a 2 week period. The reproducibility standard deviation of the HDC-ICP-MS analyses of the standards was ±20%. If we assume the same reproducibility for the HDC-ICP-MS analysis of samples, the combined reproducibility expressed as a standard deviation is ±30%. The consequence of this reproducibility standard deviation becomes most apparent when the recovery of nano-sized silica is high, as in the case of *n*-$SiO_2$.

**TABLE 1. Presence of Nano-Sized Silica in Digests of Foods with and without Silica after the Successive Stages of the *In Vitro* Digestion[a]**

| | stage of *in vitro* digestion | | | |
|---|---|---|---|---|
| food matrix | water | saliva | saliva gastric | saliva gastric intestine |
| hot water | <5 | <5 | <5 | <5 |
| hot water with SAS | 23 | 14 | 36 | 77 |
| hot water with *n*-$SiO_2$ | 67 | 104 | 26 | 104 |
| black coffee | <5 | <5 | <5 | <5 |
| black coffee with E551 | 29 | 36 ± 4 b | <5 a | 80 ± 24 c |
| black coffee with SAS | 46 | 51 ± 14 b | <5 a | 87 ± 25 c |
| black coffee with *n*-$SiO_2$ | 73 | 76 ± 29 b | 14 ± 10 a | 71 ± 26 b |
| soup | <5 | <5 | <5 | <5 |
| soup with E551 | 13 | 8 ± 2 a | <5 a | 16 ± 4 b |
| soup with SAS | 22 | 11 ± 3 a | <5 a | 38 ± 8 b |
| soup with *n*-$SiO_2$ | 83 | 84 ± 39 b | 21 ± 19 a | 98 ± 9 b |
| pancake | <5 | <5 | <5 | <5 |
| pancake with E551 | 5 | 5 ± 1 a | 9 ± 1 a[b] | 18 ± 2 b |
| pancake with SAS | 5 | 5 ± 2 a | 5 ± 1 a[b] | 14 ± 3 b |
| pancake with *n*-$SiO_2$ | 101 | 71 ± 11 b | 36 ± 11 a[b] | 84 ± 13 b |

[a] The result is expressed as a mass percentage of nano-sized silica (5−200 nm) relative to the total amount of silica in or added to the food item. For water as a matrix or digestion stage, the results are given as a single number, while for the other matrices, the result is given as the mean and standard deviation of the individual measurements. If no nano-sized silica was detected, or if the amount detected was below or comparable to the blank control samples, than the result "<5" was placed in the table, indicating lower amounts than 5%. For coffee, soup, and pancake in saliva, gastric and intestine duplicates were carried out and the complete experiment was repeated. Significantly different ($p \leq 0.05$) amounts are indicated by different letters (*e.g.*, a,b,c). [b] At the end of the gastric digestion, pH was 4 instead of 2.

Then, the result expressed as a mass percentage for individual measurements can be higher than 100% (*e.g.*, 104% in case of hot water with *n*-$SiO_2$ at the intestinal stage in Table 1).

This reproducibility of ±30% is also reflected in the relative standard deviations of the mass percentages of the individual measurements for each combination of matrix and digestion stage. The relative standard deviations vary from 6 to 46% with an average of 25%. Exceptions are the measurements of *n*-$SiO_2$ added to coffee and soup in the gastric digestion stage where the reproducibility standard deviation is of the same order as the mean. This is a result of the fact that nano-sized silica in these samples was only found in the second digestion experiment and not in the first.

As part of the sample preparation, 2 mL of subsamples of the supernatants was collected and filtered through 5 μm filters to remove fibers and large-sized materials. The filters were analyzed for total silicon content, and the results showed that the filters did not contain detectable amounts of silica. Although no attempt was made to prepare a mass balance, most of the added silica was present in the residue/supernatant in the digestion tubes.

**Water and Saliva.** The amount of nano-sized silica (5−200 nm) in coffee with E551 containing creamer at the water and the saliva digestion stage was 30 and 37%, respectively. In hot coffee with added SAS, 46 and 51% was detected as nano-sized silica, while in the water and saliva digestion without a food matrix (only hot water) to which SAS was added, 22 and 18% was determined to have a size range between 5 and 200 nm. The results for coffee are in agreement with earlier reported results for coffee creamer in hot coffee.[10] After the saliva stage (chromatograms B in Figure 4), one major peak is seen at a size range of 5−50 nm.

Powdered soup was selected as a "heavier" matrix, that is, a matrix containing more solid and fibrous material. In water and in the saliva digestion stage, the amount of nano-sized silica in soup with E551 was 13 and 8%, respectively. For soup with added SAS, comparable values of 22 and 11% were found for water and the saliva digestion stage.

Finally, pancakes were tested as a completely solid matrix. As with the soup, it was expected that this solid matrix, containing even more particulate and fibrous materials, would interfere with the presence of nano-sized silica during digestion. This was confirmed in water and saliva, as 5% of the silica in the pancake containing E551 is present as nano-sized silica. For pancake with added SAS, these percentages are also 5% for water and the saliva digestion stage.

When *n*-$SiO_2$ was added to each of the four matrices in water or the saliva digestion stage, the recovery of this material ranged from 59 to 128% in individual measurements with an average recovery of 80 ± 21%.

**Gastric Stage.** In the gastric stage (*i.e.*, after the saliva and the gastric digestion stage), no or only low levels of nano-sized silica were found in coffee with creamer containing E551 or added SAS. These levels are significantly ($p \leq 0.05$) lower compared to the levels at the saliva stage. Hot water and coffee with added *n*-$SiO_2$ also show a low recovery of nano-sized silica. Only 26 and 14 ± 10% of this fully nano-sized material was present after the gastric digestion stage, compared to the presence of 80 ± 21% of the added *n*-$SiO_2$ in the water and saliva digestion stage. These levels are significantly lower ($p = 0.05$) compared to the amounts at the saliva stage. This indicates that an important portion of the nano-sized silica is not available for detection. Digestion of coffee with creamer containing E551 at the end of the gastric stage (chromatograms C in Figure 4) produces only a relatively small signal, and since the total area is close to what can be found in some of the blank samples, it is regarded as below the quantitation limit.

As with coffee, no nano-sized silica is found in soups containing E551 or added SAS after applying the gastric stage with a pH 2. The recovery of the added *n*-$SiO_2$ is comparable with the coffee digestions, 21 ± 19% in the gastric stage. These levels are significantly ($p \leq 0.05$) lower compared to the levels at the saliva stage. The large standard deviation results from the fact that

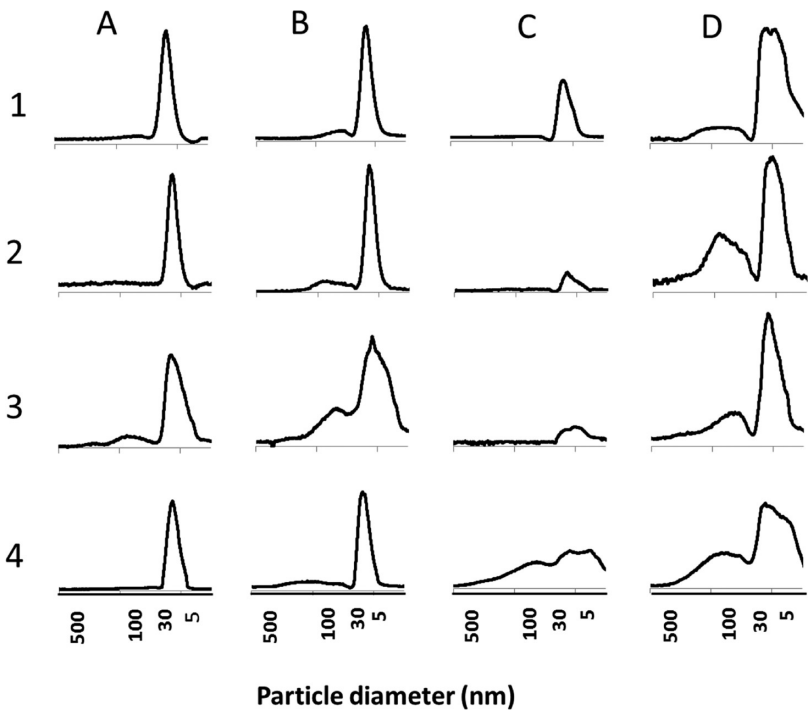


**Figure 4. Part of HDC-ICP-MS chromatograms of subsample collected from supernatant of the digestion experiments. First row, hot water with SAS; second row, coffee with E551; third row, soup with E551; fourth row, pancake with E551 in water (column A), following the saliva digestion stage (column B), the gastric digestion stage (column C), and the intestine digestion stage, or the complete digestion (column D).**

*n*-$SiO_2$ in this stage was detected only in the second series of digestion experiments and not in the first.

Different from coffee and soup, there was some nano-sized silica present after the gastric stage when pancakes with E551 and added SAS were digested. The amounts of nano-sized silica in pancake with E551 or added SAS after the gastric stage are comparable to the amounts after the saliva stage, 9 and 5%, respectively. Again, the amount of *n*-$SiO_2$, 36 ± 11%, was significantly ($p \leq 0.05$) lower than in water or the saliva digestion, although somewhat higher than in the gastric digests of coffee and soup. The probable reason for the presence of nano-sized silica in pancake is the observation that at the end of the gastric digestion stage of pancake samples the pH was not 2 but 4. This can likely be explained by the buffering capacity of the pancake matrix.

Summarizing, only low amounts of nano-sized silica were found after the gastric digestion for every food matrix with the exception of pancake where the amount was somewhat higher, probably because of the pH difference.

**Intestinal Stage.** Importantly, after completion of the full digestion procedure (*i.e.*, after the saliva, gastric, and intestine stage) the amount of nano-sized silica in coffee with E551 increased to 80 ± 24%. This means that after the complete digestion procedure a large amount of all silica is present in the size range of 5−200 nm. This result was confirmed by the complete digestion of coffee with added SAS, where 87 ± 25% of the silica added in the form of SAS was found in the size range of 5−200 nm. For both the products with E551 and added SAS, the levels after the full digestion are significantly ($p \leq 0.05$) higher compared to the levels at the saliva and gastric stage. For the *n*-$SiO_2$ addition, a recovery of 71 ± 26% was found. The *n*-$SiO_2$ levels after the full digestion are significantly higher ($p = 0.05$) than after the gastric digestion but not different from the levels present in saliva.

The chromatogram of the digestion sample of coffee with creamer containing E551 after the intestine digestion stage (chromatograms D in Figure 4), that is, after the completion of the full digestion cycle, shows at least two peaks. One peak is found at a size range of 5−50 nm, equal to the peak in chromatograms of the digestion samples after the saliva stage (chromatograms B in Figure 4), but with a different shape, suggesting a broader particle size distribution. A second and even broader peak is found at a size range of 50−500 nm. So, apart from finding more nano-sized silica after the complete digestion of coffee with creamer containing E551, we also find a shift in particle size. It appears there is an increase of particles in the size range of 5−50 nm but especially an increase of particles in the size range of 50−500 nm.

After the complete digestion of soup, the amount of nano-sized silica increased to 16 ± 4% for soup containing E551 and to 38 ± 8% for soup with added SAS. In both cases, the levels after the full digestion are significantly ($p = 0.05$) higher compared to the levels at the saliva and gastric stage. The recovery of the added

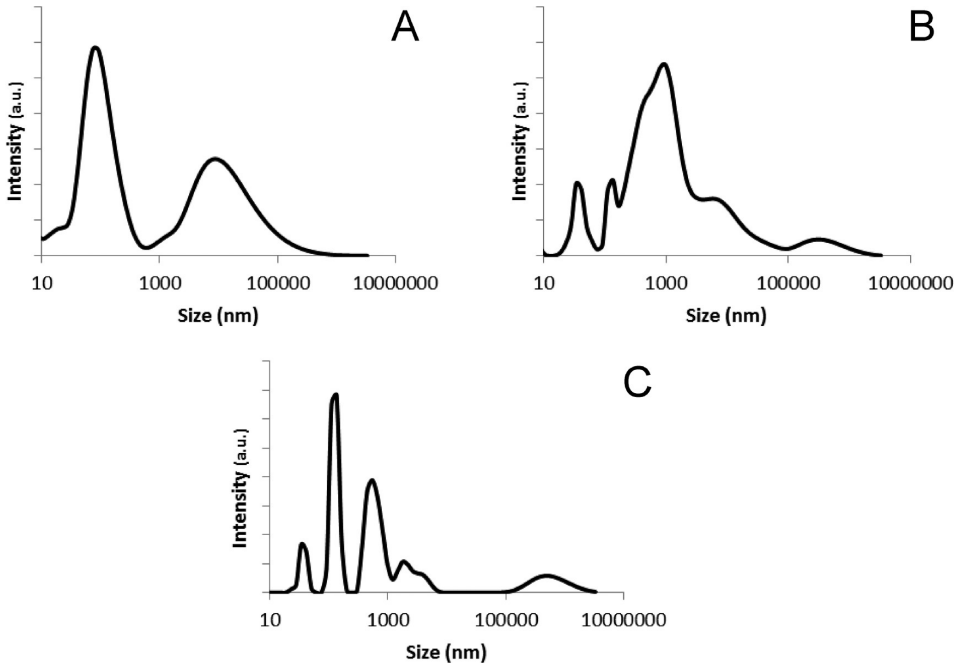


**Figure 5. Effect of pH changes on hydrodynamic size distributions of SAS dispersions in artificial saliva as determined by DLS. SAS (10 $\mu$g/mL) dispersed in the artificial saliva digestion mixture containing only inorganic components and 0.05% BSA was sonicated and incubated at 37 °C rotating head-over-heals. (A) Size distribution following 4 h incubation at pH 6.8. (B) Size distribution following 2 h incubation at pH 2. (C) Size distribution of the same suspension when pH was subsequently raised to pH 6.8.**

*n*-$SiO_2$ to soup was 98 ± 9%. The *n*-$SiO_2$ levels after the full digestion are significantly higher ($p \leq 0.05$) than after the gastric digestion but not different from the levels present in saliva.

The mass fraction of nano-sized silica in pancake increased to 18 ± 2% in the pancakes with E551 and to 14 ± 3% in the pancakes with added SAS. In both cases, the levels after the full digestion are significantly ($p \leq 0.05$) higher compared to the levels at the saliva and gastric stage. The recovery of *n*-$SiO_2$ was 84 ± 13% after the complete digestion. The *n*-$SiO_2$ levels after the full digestion are significantly higher ($p \leq 0.05$) than after the gastric digestion but not different from the levels present in saliva. The results for this solid food product shows that nano-sized silica is present following digestion if the food product contains E551 or SAS, but at lower amounts then in soup or coffee.

The high amount of nano-sized silica after the complete digestion is remarkable, given the virtual absence of nano-sized silica after the gastric stage. A disintegration of larger silica agglomerates to the primary particles and stable silica aggregates upon the transition from the gastric to the intestinal stage of digestion can explain these observations. In addition, note that bile juices are added at the start of the intestine digestion stage, and because bile juices have surfactant-like properties,[31] they may have a profound impact on the stability of nano-sized silica particles and therefore on the availability. Surfactants stabilize particles in suspension and may be important for the mobilization of particles from a matrix.[32]

**Effect of pH and High Electrolyte Concentrations on Silica Nanoparticles in the Gastric Digestion Stage.** Experiments using DLS and SEM-EDX showed that, at low pH and in the presence of high electrolyte concentration, large silica agglomerates are formed that disintegrate again when the pH is increased to neutral levels. These results indicate that the behavior of E551 and SAS upon *in vitro* digestion is in line with the extended DLVO theory.[8]

The hydrodynamic size distribution, as determined by DLS, of SAS suspended in the presence of high electrolyte concentration at pH 6.8 (*e.g.*, artificial digestion mixture containing only inorganic components resembling the saliva digestion stage) is characterized by two peaks, one with particles having sizes around 100 nm and another with a much broader size distribution around 10 $\mu$m (Figure 5A). In suspensions at pH 2, resembling the conditions of the gastric digestion, an irregularly shaped size distribution was observed, with the most prominent peak around a size of 1 $\mu$m (Figure 5B). This suggests that the smaller sized particles observed at pH 6.8 agglomerate into larger clusters and explains the low amounts of 5−200 nm sized silica particles in hot coffee and soup with E551 or added SAS after the gastric stage of the *in vitro* digestion. Upon a subsequent increase of the pH from 2 to 7, the original 100 nm peak again is the most prominent peak (Figure 5C), as was the case in the original incubation in artificial saliva. In addition, peaks around 50 and 900 nm are emerging (Figure 5C).

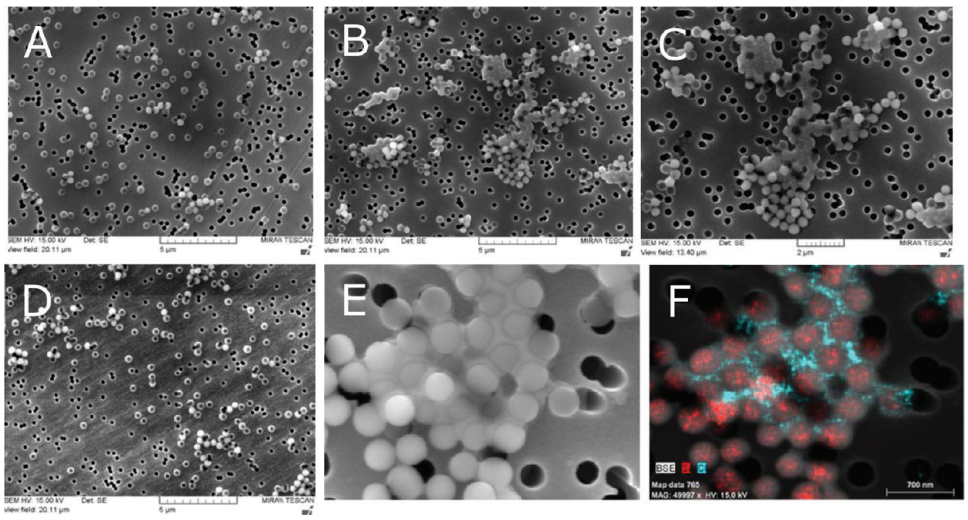


**Figure 6. SEM showing agglomeration and deagglomeration of $SiO_2$ nanoparticles due to pH changes in artificial saliva. (A) Suspension of 500 nm silica particles (white/gray dots; the dark spots are the holes in the grids) in the inorganic saliva mixture at pH 7. (B) After acidifying the suspension to pH 2, the silica particles form micro-sized agglomerates. (C) Part of image B at higher magnification. (D) After increasing the pH to 7, the original suspension is restored. (E) High magnification of micro-sized agglomerates at pH 2. (F) Same image as E with EDX showing "chloride bridges" (in blue) between the silica particles (in red).**

While dynamic light scattering is often used to assess the polydispersity of suspensions of nanoparticles, it clearly has its limitations in visualizing the nanoparticles. Therefore, we studied 500 nm silica nanoparticles in the same artificial digestion mixtures as used in the DLS measurements using SEM. Clearly separated nanoparticles are present at pH 7, as shown in a representative picture (Figure 6A). However, in the suspensions of the artificial digestion mixtures at pH 2, only a few single silica particles remain, and most are agglomerated into larger micro-sized silica agglomerates (Figure 6B). At a larger magnification (Figure 6C), "bridges" connecting the individual silica nanoparticles in the agglomerates are visible. Using EDX analysis, it was possible to show that these bridges contained mostly chlorine (Figure 6E,F). Finally, when the pH is increased to neutral pH, the image (Figure 6D) shows again separated individual nanoparticles, comparable to the image of the suspension at the start. This series of pictures shows that silica nanoparticles under acidic conditions and in the presence of electrolytes can agglomerate to micro-sized particles in a reversible manner.

**Potential Uptake of Nanosilica.** Plant-derived foods provide the highest sources of silica to humans.[11,33] Silica in the form of SAS has been used for many years in food applications as food additive E551. Dekkers *et al.*[10] reported that 4−33 mass % of the E551 in food products is in the nano-size range, and it was estimated that this would result in an intake of 1.8 mg/kg bw/day of nano-sized silica for adults, compared to 9.4 mg/kg bw/day for total silica.[10] Others reported daily dietary intakes of silica ranging from 0.1 to 60 mg/kg bw/day.[11] Soluble orthosilicic acid is suggested to be the most readily absorbable form of silica for humans,[8] with a minimum median uptake of 50%.[34] Dekkers *et al.* (2012) conclude that from the limited information on the absorption of nano-sized silica it can be assumed that nanosilica can be adsorbed from the gastrointestinal tract as nanoparticles.[35] For example, it is well-known that Peyer's patch regions play an important role in the gastrointestinal uptake of nano- and micrometer-sized particles.[36−42] However, in most reported studies, fluorescently labeled nano-sized silica particles were used, and it is unknown how the different characteristics of these particles might have influenced the absorption.[35]

On the basis of results of a rat study where the animals received feed containing 2.5 or 5% silica, EFSA concluded that the use of silica up to 1500 mg silica/day added to food supplements is of no safety concern.[43] No information on the percentage of nano-sized silica was taken into account, and a differential size-dependent uptake has not been considered.

## CONCLUSION

The dissolution, agglomeration state, and release of materials in the nano-size range from food with E551 during human digestion is a key question for the safety assessment of nanosilica. In this study, the fate of three forms of silica (E551, SAS, and 32 nm $SiO_2$) is reviewed using an *in vitro* digestion model. We used three food matrices containing E551: coffee with powdered creamer, instant soup, and pancake. Similar food products without E551 but with added synthetic amorphous silica or with added *n*-$SiO_2$ nanoparticles were included.

The results show that nano-sized (5−200 nm) silica is present in the saliva digestion stage in a relative amount of 5 to almost 40% when products containing E551 were tested. However, during the successive

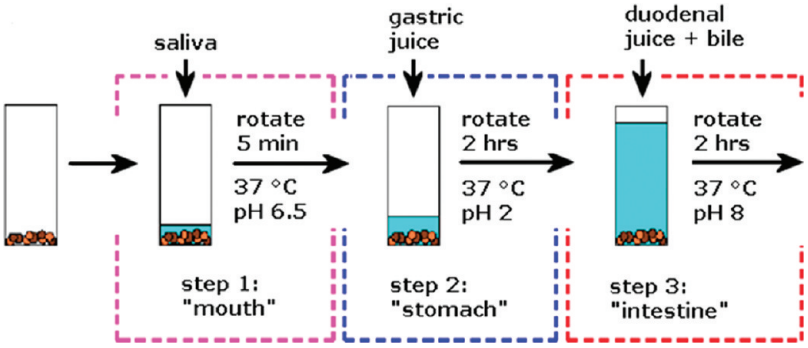


**Figure 7. Schematic presentation of the *in vitro* digestion procedure.**[27]

gastric digestion stage, this nano-sized silica disappeared in the case of coffee and instant soup, while low amounts were found for pancakes in the gastric stage. The absence of nano-sized silica is an effect of the low pH combined with high electrolyte concentrations in the gastric digestion stage. Large silica agglomerates are formed under this condition which is supported by DLS and SEM data. Importantly, when the low pH was increased to neutral pH in the intestinal stage, nano-sized silica reappears in even higher amounts than in the saliva stage. The results for coffee with E551 suggest that after complete digestion around 80% of silica is in the nano-size range. For soup and pancake with E551, that is, products containing more particulate and fibrous matter, the amount of nano-sized silica after complete digestion is around 15%

The present study clearly shows that the human intestinal wall most likely is daily exposed to nano-sized silica particles. Recently, Dekkers *et al.* concluded that single silica nanoparticles may be more easily absorbed from the human intestine, but that no conclusion on the oral bioavailability of SAS or nano-sized silica can be drawn.[35] The oral bioavailability is the key question to be resolved in further studies.

## MATERIALS AND METHODS

**Nanomaterials.** A commercially available food additive SAS with a primary particle size of 7 nm and a specific surface area of 388 $m^2/g$ from Evonik Degussa GmbH, Frankfurt, Germany, was used, kindly donated by the National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands. The 32 nm colloidal silica material (in this study referred to as *n*-$SiO_2$), stabilized at pH 8.6, was obtained from the Institute for Reference Materials and Measurements (IRMM), European Commission Joint Research Centre, Geel, Belgium. A set of silica nanoparticle suspensions with particle diameters of 100 and 500 nm was a kind gift of the University of Vienna.

**Chemicals.** All materials were cleaned carefully with Milli-Q water. All chemicals were obtained from Merck, except for $MgCl_2$, glucuronic acid, lipase (pig), and α-amylase (bacillus species) which were obtained from Sigma, and sodium *n*-dodecyl sulfate ($C_{12}H_{25}NaO_4S$) was obtained from Fluka, glucosaminehydrochloride was obtained from Calbiochem, mucin (pig) was obtained from Carl Roth (Karlsruhe, Germany), and sodium chloride and uric acid were obtained from VWR (Amsterdam, The Netherlands). Milli-Q water was prepared using a Milli-Q Gradient A10 system from Millipore, Molsheim, France.

**Selected Foods.** We selected food items in which we previously determined the fraction of nano-sized silica.[10] The food matrices used for this study were black coffee with and without powdered creamer containing E551, instant soup with and without E551, pancake powder with and without E551. To food items without E551 was added SAS or *n*-$SiO_2$. We selected these food items because of the relative high fraction of nano-sized silica and/or the relatively large portion size. Coffee was tapped from a coffee machine, while instant soup and pancakes were prepared according to the manufacturer's instructions on the package.

***In Vitro* Digestion of Food Samples.** The complete *in vitro* digestion model consists of three steps: saliva, gastric, and intestine digestion as shown in Figure 7. Three digestion experiments are started for each sample material: the first experiment is ended after saliva digestion; the second experiment is ended after saliva and gastric digestion; finally, the third experiment is stopped after saliva, gastric, and intestine digestion. After each experiment, a subsample of the supernatant is collected for analysis of nano-sized silica and the remainder of the digest centrifuged.

Artificial juices for the digestion experiments were prepared on the day before the actual digestions. The constituents and concentrations of the various synthetic juices are as shown in Table 2.

Before the incubations, all digestive juices are heated to 37 ± 2 °C and incubations are carried out in a head-over-head rotator at 37 ± 2 °C. The pH values of the digestive juices are checked and, if necessary, adjusted to the appropriate interval with NaOH (1M) or HCl (37% w/w). The digestion starts by introducing 6 mL of artificial saliva to 4.5 g of the food matrix. This mixture is rotated head-over-head for 5 min at 55 rpm at 37 ± 2 °C. Subsequently, 12 mL of gastric juice is added, the pH adjusted to pH 2.0 ± 0.5, and the mixture rotated head-over-head for 2 h. Finally, 12 mL of duodenal juice, 6 mL of bile juice, and 2 mL of $NaHCO_3$ solution are added subsequently. The mixture pH is adjusted to pH 6.5 ± 0.5, and the mixture is rotated head-over-head for another 2 h. The *in vivo* relevance for these parameters has been studied by Versantvoort *et al.*[44] At the end of the *in vitro* digestion process, the digestion tubes are positioned vertically and left in that position for 10 min at room temperature to allow large fibrous materials to settle. Next, a subsample of the supernatant is collected and filtrated through a 5 μm filter to remove large particles.

To reduce uncertainty with regard to the stability of silica nanoparticles in the filtrated supernatant of the digest, all subsamples are analyzed on nano-sized silica content within 15 min after collection. To digestion tubes containing saliva samples is added 12 mL of water because these do not always contain sufficient supernatant depending on the matrix (*e.g.*, pancake). The digest tube is shaken by hand and the content allowed to settle for 10 min before collecting the subsample. All

**TABLE 2. Composition of the Juices for Fed in Vitro Digestion Model (Amounts Based on 1000 mL of Juice)[27]**

| | saliva pH 6.8 ± 0.1 | gastric juice pH 1.3 ± 0.1 | duodenal juice pH 8.1 ± 0.1 | bile juice pH 8.2 ± 0.1 |
|---|---|---|---|---|
| inorganic constituents | •896 mg KCl<br>•200 mg KSCN<br>•1021 mg $NaH_2PO_4 \cdot H_2O$<br>•570 mg $Na_2SO_4$<br>•298 mg NaCl<br>•1694 mg $NaHCO_3$ | •2752 mg NaCl<br>•306 mg $NaH_2PO_4 \cdot H_2O$<br>•824 mg KCl<br>•302 mg $CaCl_2$<br>•6.5 mL glucose<br>•650 mg glucose<br>•20 mg glucuronic acid | •7012 mg NaCl<br>•3388 mg $NaHCO_3$<br>•80 mg $KH_2PO_4$<br>•564 mg KCl<br>•50 mg $MgCl_2 \cdot 6H_2O$<br>•180 μL HCl (37%) | •5259 mg NaCl<br>•5785 mg $NaHCO_3$<br>•376 mg KCl<br>•150 μL HCl (37%)<br>•250 mg urea<br>•167.5 mg $CaCl_2$ |
| organic constituents | •200 mg urea<br>•290 mg amylase<br>•15 mg uric acid<br>•25 mg mucin<br>•milli-Q water | •85 mg urea<br>•330 mg glucosaminhydrochloride<br>•1 g BSA<br>•2.5 g pepsin<br>•3 g mucin<br>•milli-Q water | •100 mg urea<br>•151 mg $CaCl_2$<br>•1 g BSA<br>•9 g pancreatin<br>•1.5 g lipase<br>•milli-Q water | •1.8 g BSA<br>•30 g bile<br>•milli-Q water<br><br>sodium carbonate solution<br>•84.7 g $NaHCO_3$<br>•milli-Q water |

5 μm filters and the residues of centrifuged digests were stored at −20 °C for determination of total silica.

All experiments were repeated in a second and independent experiment using the same technique and materials. In addition, in the second experiment hot water was added as a "food matrix" to determine the fate of silica in the digestion when no food sample matrix is present, and water was added as a "digestion matrix" to determine the fate of silica if no digestion liquids are present.

**Determination of Nano-Sized Silica.** Hydrodynamic chromatography coupled with inductively coupled plasma mass spectrometry (HDC-ICP-MS) is used to determine size and concentration of nano-sized silica in the samples. The HDC system is a Thermo Scientific Spectra system P-4000 liquid chromatograph (Waltham, MA, USA) equipped with a PL-PSDA HDC cartridge, type 1, length 800 mm, diameter 7.5 mm, packed with noncoated, nonporous silica spheres (Agilent Technologies, Wokingham, UK). The eluent is an aqueous 10 mM solution of sodium *n*-dodecyl sulfate (SDS) with a flow rate of 1.0 mL/min. Sample injection volumes were 50 μL. The ICP-MS is a Thermo X Series 2 (Waltham, MA, USA), equipped with an autosampler, a Babington nebulizer, and operated at an RF power of 1400 W. Data acquisition was performed in the selected ion monitoring mode monitoring *m/z* ratios of 28 and 29 that are characteristic for silicon. Polyatomic interference at these ion masses is unavoidable and resulted in a high, but relatively stable, background signal due to $N_2$. Acquiring data in the helium collision mode did not improve the signal/noise ratio and was not applied since it resulted in an overall lower sensitivity. The Si signal of peaks in the chromatograms was isolated by subtracting the background signal of the baseline in the same chromatogram. A set of silica nanoparticle suspensions ranging from 32 to 500 nm was used to calibrate the size separation of the HDC column, while a standard of 32 nm silica particles was used for quantitation and to check the performance of the system. Data are presented as a percentage of nano-sized silica relative to the total amount of silica in or added to the food item. For food items containing E551, an average silica content of 5 mg/g product is assumed based on the results determined by Dekkers *et al.*[10]

**Statistical Analysis.** For water as a matrix or digestion stage, the HDC-ICP-MS results are given as a single number, while for the other matrices, the result is given as the mean and standard deviation of the individual results. Statistical significance was determined *via* a blocked one-way analysis of variance (ANOVA). Significance levels are indicated in the table and text.

**Quality Control.** The described results were obtained in two independent series of experiments using the same materials and analytical techniques. During the first series of experiments, three food items, coffee with creamer, soup, and pancake, each without E551, with E551, with SAS, and with *n*-$SiO_2$ were digested under three conditions, saliva, gastric, and intestine. Because all digestions were carried out in duplicate, this resulted in 72 digestion experiments. To confirm the results of the first study, the entire study was repeated using the same techniques and materials but a different order of digestion experiments. During this second series of experiments, hot water was added as a "food item", and water was added as a "digestion stage" to the original setup. This resulted in a total of 96 digestion experiments in the second series.

The performance of the instrumental analysis in terms of response and retention time stability was determined as the standard deviation in the average response and retention time of the quantitation standard over the entire period of each digestion series (about two weeks). The reproducibility standard deviation of the response of the quantitation standard was ±20%, and that of the retention time <2%. The reproducibility of the response is relatively high for two reasons. First, the ICP-MS signal for Si suffers from a high background due to the presence of $N_2$, which has to be subtracted to isolate the Si signal of the analytes. Second, HDC separation does not result in clear narrow peaks as in regular gas and liquid chromatography but in broad peaks depending on the size distribution of the analyte particles. The performance of the complete experiment (*i.e.*, digestion and instrumental analysis) can be estimated from the individual results of the experiments that were performed in duplicate in both series. The reproducibility standard deviation calculated from the four individual results of each experiment and excluding "<5" results varied from ±6 to ±46% with an average of ±24%. The recovery of nano-sized silica in the digestion experiments can be estimated from those experiments that do not compromise the silica suspension stability, that is, excluding the experiments where the gastric digestion is the last step. In that case, the average recovery of the added *n*-$SiO_2$ is 83 ± 21%. The SANCO/10684/2009 document concerning method validation and quality control procedures for pesticide residues analysis in food and feed[45] states that the within lab reproducibility of a method should not exceed ±20% and that a recovery between 70 and 120% with a reproducibility of ±20% is acceptable. This means that while the recovery is acceptable the reproducibility is too high. However, it should be kept in mind that the SANCO document is applicable to well-established methods for well-defined analytes such as pesticides (single, molecular analytes) and not to the much less well-defined particulate materials as in this study. As a consequence, the quantitative performance of the method is limited.

Because nano-sized silica can also be found at low levels in tap water, the detection limit of the method was set at a value of

5 mg/L in the filtered supernatant of the digests. This equals with a detection limit of 0.2 mg/g food product.

**Determination of Total Silicon Content.** The 5 $\mu$m filters were cut open, and the actual filter was removed from the plastic housing. Digestion residues were allowed to warm to room temperature. The filters, or small amounts of the residues, were added to a PFA digestion vial followed by 6 mL of 70% $HNO_3$ and 1 mL of 40% HF. The samples were digested in a microwave system for 45 min. Following digestion and cooling to room temperature, Milli-Q water was added to a total volume of 50 mL. This solution was shortly shaken by hand and diluted two times more. Finally, the extracts were analyzed with inductively coupled plasma mass spectrometry (ICP-MS) using the same ICP-MS system and settings as described above.

**Dynamic Light Scattering.** To further assess the effect of pH on particle size and agglomeration, the SAS suspensions (10 $\mu$g/mL) stabilized with 0.05% BSA were prepared in artificial saliva juices (with only the inorganic components) at pH 7. This suspension was incubated at 37 °C for 2 h, rotating head over head. A subsample was collected and analyzed with dynamic light scattering (DLS). Subsequently, the pH of the SAS/saliva suspension was set to pH 2 and the suspension incubated for another 2 h at 37 °C (to mimic the gastric digestion stage) and analyzed again by DLS. DLS measurements were performed on an ALV laser phoniometer. All DLS measurements were conducted at a scattering angle of 90°. DLS data were processed using after ALV software.

**Electron Microscopy.** To determine the fate of the nano-sized silica particles during the gastric digestion, three suspensions of 500 nm silica particles were prepared in the saliva mixture containing only the inorganic components at pH 7. One suspension was filtered over a nickel-coated polycarbonate filter with 400 nm pores, and SEM was used to image the silica particles. The filters were analyzed with high-resolution field emission gun scanning electron microscopy in combination with energy-dispersive X-ray analysis (FEG-SEM/EDX). The microscope is a Tescan MIRA-LMH FEG-SEM (Cranberry Twp, PA, USA) operated at an accelerating voltage of 15 kV, working distance 10 mm, spot size 5 nm, and magnification 5.000−50.000 times. The EDX spectrometer is a Bruker AXS spectrometer with a Quantax 800 workstation and an XFlash 4010 detector. For this study, 500 nm silica nanoparticles instead of the 32 nm silica nanoparticles were used because of visualization limitations of SEM.

To determine the morphological characteristics of the nanoparticles, TEM pictures were made using a JEOL JEM 1011 microscope (JEOL, Nieuw Vennep, The Netherlands), delivering beam current at 045 A and fitted with a Keenview 1k × 1k camera. Grids were prepared on Cu mesh 400 holey carbon film. Nanoparticles were viewed at a concentration of 10 $\mu$g/mL to achieve optimal surface coverage. About 5 $\mu$L was dropped on and left to dry completely. The mean diameter of 10 particles is reported.

*Conflict of Interest:* The authors declare no competing financial interest.

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