1 Supporting Information for Publication: "A uniform measurement

2 expression for cross method comparison of nanoparticle aggregate

3 size distributions."

- 4 Agnieszka Dudkiewicz*1, 2, Stephan Wagner3, Angela Lehner4, Qasim Chaudhry1, Stéphane
- 5 Pietravalle¹, Karen Tiede¹, Alistair B.A. Boxall², Guenter Allmaier⁴, Dirk Tiede⁵, Ringo Grombe⁶
- 6 Frank von der Kammer³, Thilo Hofman³ and Kristian Mølhave⁷
- 7 ¹The Food and Environment Research Agency, Sand Hutton, York Y041 1LZ, UK.
- 8 ²The University of York, Heslington, York, YO10 5DD, UK.
- 9 ³Department of Environmental Geosciences, Center of Earth Sciences, University of Vienna,
- 10 Althanstrasse 14, A-1090 Vienna, AT.
- 11 ⁴Reasearch group Bio- and Polymer Analysis, Institute of Chemical Technologies and Analytics,
- 12 Vienna University of Technology, Getreidemarkt 9/164 A-1060, AT.
- ⁵Department of Geoinformatics Z_GIS, University of Salzburg, Schillerstr. 30, 5020 Salzburg,
 AT.
- ⁶Joint Research Centre, Institute for Reference Materials and Measurements, Geel 2440, Retiesweg
 111, BE.
- ⁷Deptartment of Micro and Nanotechnology, Technical University of Denmark, Lyngby 2800,
 DTU Bldg 345b, DK.
- 19 *Corresponding author, e-mail address: agnieszkaidudkiewicz@gmail.com

20 Contents

21	1. Comparison of the measurement outputs for spherical silica nanoparticles
22	Nanoparticles
23	Characterisation methods4
24	Results4
25 26	Ability of measurement methods for characterisation of sample featuring polymodal size distribution
27	Measurement method dependant differences in particle abundance through size distribution7
28	Method's particle size measurement accuracy9
29	Ability of analytical methods to detect and measure small sized silica nanoparticles
30 31	2. Definition of fractal dimension and fractal prefactor of lacunarity for studied synthetic amorphous silica sample
32	Methodology
33	Result
34 35	3. Statistical comparison of the measurement methods for particle abundance in GI, GII and GIII of K80 particle size distribution
36	Methodology16
37	Result
38 39	4. Sample preparation and imaging conditions in high vacuum and liquid scanning electron microscopy
40	Reference:
41	
42	

43 1. Comparison of the measurement outputs for spherical silica 44 nanoparticles

45 Nanoparticles

Figure A1 presents example SEM images of two kinds of spherical silica engineered nanoparticles 46 47 (ENPs) used in the study. Both ENP aqueous dispersions, defined as: K12 and K80 (commercial id 48 30V12 and 30V50 respectively) were kindly provided by AZ Electronic materials (Trosly-Breuil, 49 France). The concentration of silica in both dispersions was at the level of 30% w/w. The 50 manufacturer also provided nominal diameter of 12 nm for K12 and 80 nm for K80, which were 51 calculated from surface area (Sear's titration method). The K12 sample (Figure A1A) contained small particles and it was used for estimation of methods limits of detection in relation to silica 52 53 ENPs size (LOD_s). The K80 sample contained both smaller and larger single particles as well as some aggregates (Figure A1B) and a bimodal PNSD, which is further described in the results 54 section. This sample was used for the determination of differences between the methods for 55 quantification of smaller and larger silica ENPs within PNSD, as well as determination of method's 56 accuracy in measurement of silica ENPs size. 57



59 Figure A1. SEM images of (A) K12 and (B) K80

60 Characterisation methods

61 Same instruments as for the characterisation of SAS in main text were used. Any differences in 62 sample preparation or instrumental settings were summarised in the Table A1. Additionally 63 conditions of sample preparation as well as imaging conditions in SEM and Wet-SEM were 64 summarised in section 4.

65 Table A1. Differences in sample preparation and analysis conditions between SAS, K80 and 66 K12

Instrument	Dilution ratios	Other differences			
GEMMA	K12- 1:36.999 K80- 1:9.999	pH of ammonium acetate buffer was raised to 8.0, no filtration was applied, number of scans per sample replicate in GEMMA was increased to 10			
CLS	K12- no dilution as in case of SAS K80- 1:9 in BB8.0	Same conditions as in case of SAS			
NTA	K12- 1:5 999 K80- 1: 999 999	Same conditions as in case of SAS			

67

68 **Results**

The particle size distributions derived from different techniques are given in Figure A1. The PNSD 69 of K80 was divided in 3 size groups chosen based on minimal particle count in between ENP 70 71 populations as given by SEM: primary particles: smaller: group I (18-62 nm), larger: group II (63-106 nm) and agglomerates: group III (107-160 nm). These groups served for comparison of relative 72 particle abundance within the PNSD between the methods by the statistical evaluation described in 73 74 this Supporting Information section 3. The comparison of modal size measurements from all the techniques against SEM was used here to define accuracy of the techniques, as electron microscopy 75 has been applied in most research papers for characterisation of ENPs¹ and is recommended as a 76 77 reference method for particle size measurement ^{2, 3}.

78 Ability of measurement methods for characterisation of sample featuring

79 polymodal size distribution

Two out of the six methods (NTA and Wet-SEM) did not provide a level of resolution necessary to distinguish the two particle populations in K80 PNSD (Figure A2). For NTA, similar results were previously reported ^{4, 5}. Two hypotheses can be used to explain why Wet-SEM did not provide sufficient resolution between the two particle size populations:

Beam broadening during membrane passage and in aqueous environment might have an
 effect on the particle size in the same way as positioning of specimen out of focus

- Subsequent image analysis of blurred and low contrast images as produced by Wet-SEM
 images (Figure A7) might be inaccurate. Therefore it is possible that the error introduced by
 the image analysis is simply too large to deliver reliable image statistics.
- 3. The remaining four methods SEM, GEMMA, CLS and AF4-ICP-MS were able to resolve
- 90 the bimodal PNSD, but exhibited size related differences in particle abundance (see section
- 91 3 below).





92 93 Figure A2. Number-size distribution of K80 and K12 samples measured by different techniques, K80-measurments given as total particle size distribution from 3 replicates (SEM, NTA, Wet-94 95 SEM), mean (GEMMA) or selected single replicate (CLS, AF4-ICP-MS), K12- measurements from 3 replicates shown (and 2 replicates for Wet-SEM)- different colours show different replicates 96

97 Measurement method dependant differences in particle abundance through

98 size distribution

- 99 Table A2 provides a summary of modal and mean measured particle diameters in determined
- 100 points of the PNSD and relative particle abundance in predetermined size groups I-III.
- 101 Table A2. Mean and modal diameters of particles in the size distribution of K80 and relative

102 abundance of particles in predetermined size groups (I-III)

Method	Size (s.d.)			Relative particle abundance (%)			
	Mean	Mode I	Mode II	Ι	II	III	
SEM	76 (3) ^{AB}	47 (2)	85 (1)	48	36	16	
GEMMA	69 (1) ^C	46 (1)	87 (2)	41	58	1	
CLS	77 (1) ^{AB}	44 (0)*	84 (1)	20	79	1	
AF4-ICP-MS	76 (1) ^{ABC}	51 (1)*	81 (1)*	23	74	3	
NTA	82 (2) ^A	N/A	N/A	22	62	16	
Wet-SEM	74.6 (5.6) ^{BC}	N/A	N/A	26	64	10	

^{A-C}Same letter in column with mean size value of K80 sample marks that no significant difference was detected between measurement methods (Tukey's test, p>0.05), *Significant difference detected in comparison to SEM measurement (Dunnett's test, p<0.05).

107 The small particles in group I of the PNSD were found most abundant in SEM (48%) and least 108 abundant in CLS and AF4-ICP-MS (20% and 24% respectively). Aggregates (group III) were also 109 most abundant in PNSD derived by SEM (16%) when compared to GEMMA, CLS (1% both) and 110 AF4-ICP-MS (3%). These differences had obvious effect on the particle mean size. CLS, SEM and AF4-ICP-MS mean diameter measurements (77, 76 and 76 nm respectively) were not significantly 111 different from each other (Tukey's test, p > 0.05) and comparable to declared by manufacturer 112 113 diameter (80 nm). The GEMMA derived mean diameter was significantly smaller at 69 nm (with exception of AF4-ICP-MS). 114

115 However, it is unclear which of the methods measured the sample more accurately. In case of SEM,

116 sample preparation might introduce particle agglomeration on the substrate ⁶⁻⁸. The sample

117 preparation chosen here was limiting particle agglomeration compared to others (see section 4),

however it is believed that this artefact cannot be completely avoided as the particles are transferred from the relatively large suspension volume onto two dimensional surface of a substrate. Limitation of space on the substrate will cause eventual overlap of some particles. Agglomeration also occurs in the concentrated particle dispersions with passing time naturally ⁹. Therefore, factors like dilution ratios and time from suspension preparation to analysis which were varying in between the methods used in this study could have an effect on the agglomeration state of the ENPs.

124 Another microscopy sample preparation artefact suggested in previous studies was specificity to different particle size fractions- selective retention of smaller ENPs or/and loss of larger ENPs^{8, 10}. 125 126 The CLS method was previously reported to closely follow particle number proportions in PNSD to the expected values ⁵ and in this study also provided similar particle abundance proportions in 127 128 group I and II as AF4-ICP-MS (see Supporting Information, section 4 for statistical evaluation). 129 Nevertheless analysing measurement output from AF4-ICP-MS, we have found that data points for K80 ENPs below 39 nm were below estimated concentration limit of detection for ICP-MS (10 µg/ 130 131 L). Therefore it is expected that number concentration of ENPs in group I was underestimated by 132 AF4-ICP-MS.

Available data in the published literature suggest that GEMMA could provide accurate 133 134 quantification of particles through PNSD. GEMMA's condensation particle counter unit features 135 exponential growth to maximum of particle registration efficiency from smaller to larger sizes. It 136 has been shown that for the used model the near to maximal registration efficiency (approximately 137 0.95) was obtained after reaching EMD of 4 nm for silver ENPs ¹¹. Furthermore data are also available on the determination of monomodal PNSD of spherical gold ENPs by several analytical 138 methods including GEMMA and transmission electron microscope (TEM) ¹²⁻¹⁴. These publications 139 140 reported, that the PNSDs of Au ENPs generated by GEMMA and TEM had similar shape and size 141 range, thus there is no evidence that GEMMA is biased toward quantification of the particles142 characterised by smaller or larger sizes.

143 Method's particle size measurement accuracy

The particle modal diameters in K80 determined by GEMMA (Mode I: 46, Mode II: 87) were not 144 145 significantly different to SEM measurements (Mode I: 47, Mode II: 85 nm, Dunnett's test, p > 0.05), providing more confidence that GEMMA measurements were accurate. CLS results also compared 146 147 well to SEM measured particle size in Mode II (84 nm, Dunnett's test, p>0.05), but significantly smaller in Mode I (44 nm, Dunnett's test, p < 0.05). However, AF4-ICP-MS showed particles larger 148 149 in Mode I (51 nm) and smaller in Mode II (81 nm, Dunnett's test, p < 0.05) than SEM. Although 150 size differences between SEM and AF4-ICP-MS or CLS were small (less than 10% of the SEM 151 measured diameter), given that for AF4-ICP-MS and CLS original data outputs are particle mass 152 and intensity weighted size distributions transferred by calculation into PNSD, some degree of 153 approximation in calculated particle number was expected. This issue was further discussed in the Supporting Information, section 3. 154

155 Ability of analytical methods to detect and measure small sized silica 156 nanoparticles

157 We found that neither of the methods studied, provided size measurement for K12 sample close to158 the nominal 12 nm given by the manufacturer (Table A3).

Method	Min ^a	Mode (s.d.)	5% (s.d.)	50% (s.d.)	95% (s.d.)
SEM	8.0	15 (2)	11 (0)	19(1)	38 (3)
GEMMA	8.0	23 (2)	12 (1)	22 (0)	40 (1)
CLS	9(1)	15 (7)	11 (2)	21 (6)	50 (9)
AF4-ICP-MS	7 (0)	27 (1)	18 (0)*	28 (1)*	44 (0)
NTA	23 (1)	70(1)	42 (0)*	77 (1)*	152 (5)*
Wet-SEM	38 (1)	94 (1)	60 (5)*	100 (4)*	174 (15)*

160 Table A3. Mode and percentile diameter in the particle number size distribution of K12

^aDiameter of smallest detected particle, except SEM and GEMMA where the values represent cutoff point accepted for the analysis, *Significant difference detected in comparison to SEM measurement (Dunnett's test, p < 0.05).

It became apparent that Wet-SEM and NTA did not allow to measure the main population of ENPs 165 166 in K12 sample (see Figure A2), and instead detected only larger particles (median: 77 and 100 nm 167 respectively). The presence of these large particles was not reflected in PNSD delivered by other methods. The CLS showed a very high measurement uncertainty noticeable between replicates 168 169 (Figure A2), reflected by a high standard deviation values for subsequent percentiles (Table A4). Interestingly, a previous study with the same model of instrument and setup was able to obtain very 170 reproducible results for silica ENPs with 20 nm modal diameter (expanded uncertainty <5%)¹⁵. 171 172 The K12 ENPs studied here were characterised by a slightly smaller diameter (15 nm by SEM). This slight difference in particle size prolonged the time needed to analyse the sample. Analysis of 173 one replicate took 1.5 hours which is longer than recommended by the instrument manufacturer. 174 175 Additionally we observed that one of the replicates, which was analysed as the last one displayed 176 better peak shape, more resembling PNSD shown by other techniques (blue graph on Figure A2). 177 This observation indicates that technique can be perhaps optimized for the measurement of very fine silica ENPs. Nevertheless required sample concentration for the measurement of such ENPs in 178 CLS must be very high (here 30%w/w) and obtained PNSD interpreted with caution since detector 179 180 seems to lose of sensitivity for fine silica ENPs after certain size point as shown for K80 sample.

181 Contrary to what was found previously for K80 sample, K12 sample was analysed by SEM without182 apparent agglomeration introduced by sample preparation.

The use of the K12 sample was aimed at testing the LOD_s. However, accurate estimation of LOD_s 183 184 for most of the methods was not possible. For SEM, a cut-off point (8 nm) was based on image pixilation and a thickness of conductive coating (4 nm- see Supporting Information, section 4), as 185 186 for particles with radius smaller than the thickness of the coating, only a part of the particle could 187 be visualized. The smallest observed particles were smaller than 8 nm and hence hindered the exact LOD_s characterisation. In GEMMA an additional peak starting at approximately 8 nm and 188 189 continuing toward lower size values was observed in PNSD. This peak was attributed to the 190 background noise (presence of non-volatile substances in the sample e.g. dissolved SiO₂, or Na 191 salts) and was also noticed during examination of the K80 sample (starting at 10-14 nm therefore 192 not shown in the Figure A2). Thus the LOD_s in GEMMA was dependent on the level of contamination in the test sample. 193

194 It was not possible to accurately estimate the size LOD_s for Wet-SEM either. The observed 195 minimal particle size was 38 nm, which was above the set cut-off point for the K12 sample analysis 196 (see Table A4). However, for the K80 sample, imaging at higher magnification was possible (see 197 Table A4) and particles down to 27 nm diameter could be detected. This indicated that the size 198 LOD_s in Wet-SEM was affected by particles drifting away from the membrane at higher 199 magnifications (see Supporting Information section 4).

The CLS detected particles at or close to 8 nm cut-off for the two replicates but for one replicate the size distribution terminated at 11 nm (see Figure A2). Therefore the LOD_s could not be unambiguously determined. It should be mentioned that in this study we used a very concentrated K12 dispersion (30% mass of SiO₂). At 10 fold dilution, no response could be detected from this

204 sample (data not shown) and therefore it could be concluded that in fact the majority of the 205 particles in PNSD of K12 was under the LOD_s of CLS.

For the AF4-ICP-MS and NTA, the smallest measured particle size could be clearly identified (7 and 23 nm respectively). Thus silica LOD_s for NTA was identified. However, for AF4-ICP-MS this value could be even lower than the smallest detected particle size, as we were not able to confirm with any other technique that particles smaller than 7 nm were not present in the sample.

Interestingly, we also found that PNSD of K12 from NTA and Wet-SEM before reaching the size 210 of the smallest detected particles, displayed a gradual decay of particle abundance rather than rapid 211 212 cut-off particle population as for example previously reported in study on single particle ICP-MS ¹⁶. This result suggested that some analytical methods for ENPs analysis may not only be bound by 213 214 the size LOD_s but also limit of quantification for particle size (LOQ_s)- size below which particle 215 count is no longer accurate. The LOQ_s was likely a reason for detection of significant differences in particle abundance in groups I-III of K80 PNSDs (see Supporting Information, section 3 for 216 217 statistical analysis).

219

2. Definition of fractal dimension and fractal prefactor of

220

lacunarity for studied synthetic amorphous silica sample

221

The studied material- synthetic amorphous silica (SAS) has an agglomerated structure that can be characterised as a fractal aggregate as described by Boldridge ¹⁷. The main parameters are the fractal dimension (D_f) and fractal prefactor of lacunarity (k_0), that relates the number of primary particles in an aggregate with the primary particle diameter and radius of gyration (R_g) through Eq. 1 given in the article.

227 Methodology

The modal primary particle diameter (d_{pp} =9 nm) value was obtained from the size distribution of non-aggregated residual single particles and this single value was further used for calculations. Cross-check of accuracy of measured d_{pp} was achieved by comparing specific surface area (SSA) given for a precursor powder for measured here SAS dispersion and SSA calculated based on our measured d_{pp} and comparison with data for SAS from other publication ¹⁷. The SSA reported for precursor SAS powder was 226 m²/g and calculated from measured d_{pp} according to Eq. A1, SSA=303 m²/g.

$$SSA = \frac{6}{d_{pp}\rho_{SiO_2}}$$
 Eq. A1

235

The SSA of aggregates is typically smaller than joint SSA of particles creating aggregate ¹⁸ ¹⁹. How much smaller depends on the area with which the primary particles are fused together making it inaccessible for gas adsorption (method used for the SSA measurement).

239 In one of the references given in the paper ¹⁷ authors provided fractal characterisation of silica 240 fumed powder with SSA=90 m²/g. The geometric mean primary particle size they obtained from transmission electron microscopy images was 22.5 nm and this gives SSA of 121 m²/g. It can be noticed that the quotient of calculated and measured SSA of silica aggregates is very similar in our and the cited study (1.34 and 1.35). Therefore we believe that this quotient is a factor that should be used to correct for the non accessible for the gas adsorption part of silica fumed aggregates in SSA estimation from primary particles. This would also mean that our primary particle measurement was accurate.

247

248 Determination of SAS fractal characteristics was done according to previously described 249 dependencies ¹⁷.

In total 3791 SAS aggregates were measured. If the ECD of measured SAS particle was $\leq d_{pp}$, it was assumed that the number of primary particles within measured aggregate (*N*) was equal to 1. In larger SAS aggregates the number of primary particles was calculated according to Eq. 3 provided in the article. The D_f and k_0 were derived from the slope of linear regression of ln (*N*) on ln (d_{max}/d_{pp}) (Figure A2). The d_{max} was Ferret's diameter directly measured from the SEM images and reduced by 8 nm to correct for the conductive coating thickness (see Supporting Information, section 4).

257 Result

258 The plot of ln (N) from ln (d_{max}/d_{pp}) is presented in Figure A3.





The D_f was equal to the slope of the linear curve (2.11) and k_0 was calculated from Eq. A2 ¹⁷ and gave value of 1.17.

263

$$k_0 = 0.69^{-D_f} e^b$$
 Eq. A2

264

265 Where:

266 *e*- mathematical constant

267 b- intercept of the linear regression on the log-log scale

268

269 The generated curve was based on the measurement of all aggregates in 3 sample replicates. The

270 maximum difference in between replicates in estimate of D_f was 0.06 and k_0 0.08.

3. Statistical comparison of the measurement methods for particle abundance in GI, GII and GIII of K80 particle size distribution

275 Methodology

The % abundance of particles in groups I-III in Figure A2 and Table A2 in the first section was an 276 example of compositional data. Such data can be only subjected to statistical analyses after one of 277 the transformations described by Aitchinson ²⁰. This is due to the sum-to-one constraint which 278 279 makes variables dependent on each other. Here we have used the additive log-ratio (alr) 280 transformation because it allows to simplify the interpretation of the statistical analysis in comparison to other available transformations ²¹. The transformed data were obtained by dividing 281 282 the relative particle number in group I and group III by relative particle number in group II and acquiring natural logarithms for the respective quotients. The group II was used as a denominator 283 because the % particle abundance deviated on average less between data from different methods in 284 285 comparison to group I and III. This was done for ease of the data interpretation.

Because of the data transformation nature it is not possible to conclude from the statistical analysis, which variable given in the nominator or denominator of the natural logarithm is responsible for the detected difference. However, low variability of measurement used as a denominator puts more confidence into significance of nominator's contribution. The statistical tests outcomes were discussed by the comparison with the original data accordingly.

291 The statistical significance of differences in particle abundance between size groups from different 292 methods was determined using MANOVA. To determine which pair of groups the difference came 293 from (ln(group I/group II) or ln(group III/group II)) two separate ANOVAs were run. To find out

which of the methods generated different particle abundance in size groups Tukey post-hoc tests were run for both ANOVAs. The significance level for all tests was $p \le 0.05$. All tests were performed using the IBM SPSS Statistics 21 software.

297 Result

The MANOVA test proved that there was a statistically significant difference in between the methods regarding reported particle abundance in tested size groups I-III (F(10, 20)=45.78, p<0.05).



301 The results of further statistical comparison of *alr* transformed data are summarised in Figure A4.

302

Figure A4. The data for statistical comparison of particle abundance in group I-III of K80 between
measurement methods. Mean values and standard deviation (error bars) of *alr* transformed relative
particle counts in particle size groups.

306 There were only two methods between which no difference was detected in both ln(group I/group

307~ II) and ln(group III/ group II) namely NTA and Wet-SEM.

SEM revealed higher proportions of particles than GEMMA, CLS and AF4-ICP-MS in both group I and group III (see Table A2). It is anticipated that the used sample preparation method for SEM could be specific to small particles and additionally caused particle agglomeration. However, the value of ln(group III/ group II) between SEM, NTA and Wet-SEM occurred to be not significantly different, and indeed particle % abundance in group III for NTA and Wet-SEM (16 and 10% respectively) was similar to obtained for SEM (16%). This could be associated with poor resolution of the NTA and Wet-SEM for distinct particle populations in group I-III (see Figure A2).

The particle abundance in group I and group II of AF4-ICP-MS measured K80 PNSD (24 and 73% respectively) were very similar to CLS (group I: 20%, group II: 79%) and thus no difference was detected in ln(group I/ group II) between these methods. However, the % abundance of group III aggregates was three times higher in K80 PSND from AF4-ICP-MS (3%) when compared to K80 PSND from CLS (1%) and significant difference was detected in ln(group III/ group II). This finding may mean that particles of K80 agglomerated slightly in AF4 eluent or could be a consequence of sample analysis at different time in AF4-ICP-MS and CLS facility.

322 Due to detected difference in K80 particle modal size between SEM when compared to CLS and 323 AF4-ICP-MS (see Supporting Information, section 1) another statistical analysis was performed 324 correcting the particle abundance in group I and group II for the size measurement inaccuracy. This 325 correction was important for CLS and AF4-ICP-MS because the PNSDs were calculated from 326 initially mass based particle size distributions. The correction was made based only on modal 327 particle size values in group I and group II according to Eq. A3.

$$N_{corr} = N_{cal} \times \left(\frac{d_{cal}}{d_{corr}}\right)^3$$
 Eq. A3

328 Where:

329 Ncorr- Corrected particle number

- 330 N_{cal} Calculated particle number in mass to number conversion
- 331 d_{cal} Measured modal particle diameter in the distribution
- 332 d_{corr} Accurate modal particle diameter in the distribution (SEM measurement)
- 333 The correction resulted in average increase of K80 particle abundance in group I by 7% and same
- 334 decrease in group II for AF4-ICP-MS. For CLS the particle abundance decreased on average in
- 335 group I by 3% and increased by 3% in group II.
- 336 The results of repeated statistical analysis for recalculated particle abundance for AF4-ICP-MS and



337 CLS were summarised in Figure A5.

338

Figure A5. The data for statistical comparison of particle abundance in group I-III of K80 between measurement methods where AF4-ICP-MS and CLS % particle abundance in group I and group II was corrected for size measurement error in relation to SEM. Mean values and standard deviation (error bars) of *alr* transformed relative particle counts in particle size groups.

Only one change in statistical analysis was obtained due to CLS and AF4-ICP-MS data adjustment.
The value of ln(group I/group II) for CLS and AF4-ICP-MS this time occurred to be statistically
significantly different.

- 347
- 348

349

4. Sample preparation and imaging conditions in high vacuum and liquid scanning electron microscopy

The sample preparation method was chosen after in-house performed evaluation of several protocols based on either electrostatic deposition, sedimentation in the ultracentrifuge or drying of the silica ENP suspensions on the TEM grid. In course of this evaluation it became clear that electrostatic deposition method compared to sedimentation and drying methods allowed to minimise silica ENPs agglomeration. Hence the samples for high vacuum SEM were prepared by electrostatic deposition of ENPs on gelatin coated TEM grid (Formvar-carbon coated from Agar Scientific- Stansted, UK).

For coating, the grids were placed on a drop of 0.1% freshly prepared solution of gelatin from 357 porcine skin type A (G6144-100G, Sigma Aldrich- Dorset, UK) for 5 min and washed in 3 drops of 358 359 demineralized water. Coated grids were placed in contact with the samples for 2 min and washed in 360 2 drops of demineralized water. The excess sample and water was blotted off using filter paper to the point of visual dryness. The dried grids were attached to the aluminum SEM stubs using carbon 361 tape and coated with Pt/Pd prior to imaging using a sputter coater (JEOL JFC-2300HR High 362 Resolution Fine Coater with a JEOL FC-TM20 Thickness Controller). The coating was expected to 363 increase particle size. This increase was estimated experimentally (measuring ENPs before and 364 after coating) and subtracted from all measurements. Increase in particle ECD was 8 nm for 365

366 spherical ENPs and SAS ENPs smaller than 116 nm. For SAS ENPs with ECD>116 nm (measured

367 including coating) size increase was following linear dependence (Eq. A4).

$$\Delta ECD = 0.14 \times ECD_m - 8.2$$
 Eq. A4

368 Where:

- 369 $\triangle ECD$ ECD increase due to conductive coating
- 370 ECD_m ECD measured after coating

371 Capsules for Wet-SEM imaging were treated with 0.1% solution of poly-l-lysine (P8920, Sigma 372 Aldrich, UK) prior to sample application according to the protocol available on the manufacturer's 373 website ²².

Prior to preparation for SEM and Wet-SEM imaging all samples were diluted with BB8.0 in ratio 374 indicated in Table A4. The zeta potential of the ENPs in the BB8.0 was measured to assess their 375 stability using electrophoretic light scattering (Zetasizer Nano ZS, Malvern, Worcestershire, UK). 376 377 The measurements were preformed on samples diluted in ratio 1:9 (K12 and SAS) and 1:99 (K80). For K12 and K80 zeta potential was -30.5±1.7 mV -and -33.4±0.7 mV respectively and for SAS -378 29.9 mV±1.4 mV. These values indicate that all the ENPs were stable in BB8.0 used for dilutions. 379 The smallest measurable particle ECD-cut-off point for SEM images was assessed experimentally 380 taking images of same sample particles at varying magnifications and analysing these sample 381 images using image analysis software. 382

Sample K12		K80		SAS		
Method	SEM	Wet- SEM	SEM	Wet- SEM	SEM	Wet- SEM
No of replicates	3	2	3	2	3	2
Dilution ratio	1:149.999	1:9	1:1999	1:9	1:399	1:1
Single micrograph area (µm ²)	3.98	29.14	13.06	13.06	3.98	29.14
Pixel edge (nm)	3.4	8.6	5.7	5.7	3.4	8.6
Smallest measurable particle- ECD (nm)	8	38	13	21	8	38
Number of particles measured per replicate- mean (s.d.)	465 (129)	393 (245)	1165 (342)	544 (398)	1291 (628)	740 (268)

384 Table A4. Data acquisition properties for SEM and Wet-SEM

385

In Wet-SEM imaging setup particle visibility could be improved by exposure of the capsule membrane to the electron beam for approximately 1 min at low 5000x magnification. It was noted that with time of the beam exposure particles were gradually attracted closer to the capsule membrane, increasingly populating the irradiated area (Figure A6A and B). The particles were apparently moving at the increased magnification required for the measurement consequently creating zig-zag patterns on the images (Figure A6C). Therefore only objects which looked approximately spherical were measured.

393 Example Wet-SEM images used for the measurement of silica ENPs in respective samples are394 presented in Figure A7.



- **Figure A6**. Wet-SEM images of silica ENPs (A)- taken at the start point, (B)- same area as (A)
- 397 after 1min exposure to the beam, (C)- movement of attracted particles at increased 398 magnification



400 Figure A7. Wet-SEM images of silica ENPs (A)- K12, (B)- K80, (C)- SAS (scale bar on every
401 image is 1000 nm)

- 402 The particles of K12 and K80 had visibly lower contrast to SAS. Additionally we observed that it
- 403 was not possible to discriminate between shape of spherical and SAS ENPs.

404 **Reference:**

- 405 1 L. Calzolai, D. Gilliland and F. Rossi, *Food Addit. Contam. A*, 2012, 29, 1183–1193.
- 406 2 EFSA Scientific Committee, EFSA Journal, 2011, 9(5):2140, 1–36.
- 407 3 T. P. J. Linsinger, G. Roebben, D. Gilliland, L. Calzolai, F. Rossi, N. Gibson and C. Klein, 2012,
- 408 http://publications.jrc.ec.europa.eu/repository/bitstream/11111111126399/2/irmm_nanomaterial
- 409 s%20%28online%29.pdf, (accessed June 2014).
- 410 4 D. Mahl, J. Diendorf, W. Meyer-Zaika and M. Epple, Colloid. Surface. A, 2011, 377, 386–392.
- 411 5 W. Anderson, D. Kozak, V. A. Coleman, Å. K. Jämting and M. Trau, *J. Colloid and Interf. Sci.*,
 412 2013, 405, 322–330.
- 413 6 F. J. Doucet, J. R. Lead, L. Maguire, E. P. Achterberg and G. E. Millward, *J. Environ. Monitor.*,
 414 2005, 7, 115.

- 415 7 K. Tiede, A. Boxall, S. Tear, J. Lewis, H. David and M. Hassellov, *Food Addit. Contam. A*,
 416 2008, 25, 795–821.
- 417 8 P. Luo, I. Morrison, A. Dudkiewicz, K. Tiede, E. Boyes, P. O'Toole, S. Park and A. B. Boxall, J
- 418 *Microsc*, 2013, **250**, 32–41.
- 419 9 M. Kobayashi, F. Juillerat, P. Galletto, P. Bowen and M. Borkovec, *Langmuir*, 2005, 21, 5761420 5769.
- 421 10 E. Balnois and K. J. Wilkinson, Colloid. Surface. A, 2002, 207, 229-242.
- 422 11 J. Kesten, A. Reineking and J. Porstendörfer, Aerosol Sci. Technol., 1991, 15, 107–111.
- 423 12 J. A. Small and R. L. Watters, 2012, <u>http://www.dunesciences.com/files/SRM8011.pdf</u>,
 424 (accessed June 2014).
- 425 13 D. L. Kaiser and R. L. Watters, 2007, <u>http://www.dunesciences.com/files/SRM8012.pdf</u>,
 426 (accessed June 2014).
- 427 14D. L. Kaiser and R. L. Watters, 2007, <u>http://www.dunesciences.com/files/SRM8013.pdf</u>,
 428 (accessed June 2014).
- 429 15A. Braun, V. Kestens, K. Franks, G. Roebben, A. Lamberty and T. P. J. Linsinger, *J. Nanopart.*430 *Res.*, 2012, 14, 1–12.
- 431 16F. Laborda, J. Jiménez-Lamana, E. Bolea and J. R. Castillo, *J. Anal. At. Spectrom.*, 2013, 28,
 432 1220–1232.
- 433 17D. Boldridge, Aerosol Sci. Technol., 2010, 44, 182–186.
- 434 18D. Walter, in *Nanomaterials Report*, ed. Deutsche Forschungemeinschaft, Wiley-VCH, Bonn,
 435 2013, ch.1.1, pp. 9–24.
- 436 19ISO/TS 27687:2008 Nanotechnologies- Terminology and definitions for nano-objects437 Nanoparticle, nanofibre and nanoplate.

Aitchinson,

- 439 <u>http://www.leg.ufpr.br/lib/exe/fetch.php/pessoais:abtmartins:a_concise_guide_to_compositional</u>
- 440 <u>data_analysis.pdf</u>, (accessed March 2014).
- 441 21 J. Aitchinson, 2008, http://dugi-doc.udg.edu/handle/10256/706, (accessed June 2014).
- 442 22 Quantomix, 2011, http://www.quantomix.com/, (accessed September 2011).