National Institute of Standards & Technology

Report of Investigation

Reference Material 8011

Gold Nanoparticles
(Nominal 10 nm Diameter)

This Reference Material (RM) is intended primarily to evaluate and qualify methodology and/or instrument performance for the physical/dimensional characterization of primary nanoscale particles. The suspension contains primary particles (monomers) and clusters of primary particles. RM 8011 consists of nominally 5 mL of citrate-stabilized Gold (Au) nanoparticles in an aqueous suspension, supplied in hermetically sealed pre-scored glass ampoules sterilized by gamma irradiation. A unit of RM 8011 consists of two 5 mL ampoules.

Limitations of Use: RM 8011 has been determined to contain clusters and larger aggregates of primary particles and the use is limited to methods that measure primary particle size. These methods are listed in Table 1 below. RM 8011 is not suitable for use with ensemble methods that are affected by the presence of aggregates.

Expiration of Value Assignment: The reference values for **RM 8011** are valid, within the measurement uncertainty specified, until **25 October 2020**, provided the RM is handled and stored in accordance with the instructions given in this report (see "Notice and Warning to Users"). This report is nullified if the RM is damaged, contaminated, or otherwise modified.

Maintenance of RM: NIST will monitor this RM over the period of its validity. If substantive technical changes occur that affect the reference values before the expiration of this report, NIST will notify the purchaser. Registration (see attached sheet or register online) will facilitate notification.

Overall technical coordination for material procurement, processing, and measurement activities was conducted by V.A. Hackley and J.F. Kelly of the NIST division formerly known as the Ceramics Division.

Reference and informational value measurements were performed at NIST by the following: T.A. Butler, R. Case, K.W. Pratt, L.C. Sander, M.R. Winchester, A.J. Allen, T.J. Cho, J. Grobelny, V.A. Hackley, D.-I. Kim, P. Namboodiri, J.E. Bonevich, A.J. Shapiro, M.L. Becker, D.L. Ho, A. Karim, B.M. Vogel, B. Ming, A.E. Vladár, L.F. Pease III, M.J. Tarlov, D.H. Tsai, M.R. Zachariah, and R.A. Zangmeister.

Statistical consultation on measurement design and analysis of the reference value data were performed by A.I. Avilés of the NIST Statistical Engineering Division.

Additional technical and coordination aspects were provided by the following: R.F. Cook, W.K. Haller and D.L. Kaiser of the NIST Materials Measurement Science Division.

Support aspects involved in the issuance of this RM were coordinated through the NIST Office of Reference Materials.

RM 8011 was developed at the request of the National Cancer Institute (NCI). Development and production costs were subsidized by NCI.

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Report Revision History on Last Page

Reference Values: Reference values are a best estimate of the true value provided by NIST where all known or suspected sources of bias have not been fully investigated by NIST [1]. Dimensional reference values (mean particle diameter in solution, as an aerosol and deposited on a substrate) are reported and are based on the following measurement techniques: atomic force microscopy (AFM), scanning electron microscopy (SEM), transmission electron microscopy (TEM) and electrospray-differential mobility analysis (ES-DMA). The corresponding reference values and expanded uncertainties are provided in Table 1. A synopsis of the methods used to generate reference values is provided starting on page 6. The measurands are the particle size based on the indicated methods. The reference values are metrologically traceable to the SI unit for length, expressed as nanometers.

Table 1. Reference Value Mean Size and Expanded Uncertainty [(a)]
Average Particle Size (Diameter), in nanometers

Technique	Analyte Form	Particle Size (nm)
Atomic Force Microscopy	dry, deposited on substrate	8.5 ± 0.3
Scanning Electron Microscopy	dry, deposited on substrate	9.9 ± 0.1
Transmission Electron Microscopy	dry, deposited on substrate	8.9 ± 0.1
Differential Mobility Analysis	dry, aerosol	11.3 ± 0.1

[(a)] The expanded uncertainties, U, are calculated as $U = ku_c$, where u_c is intended to represent, at the level of one standard deviation, the combined standard uncertainty calculated according to the ISO/JCGM Guide [2]. The coverage factor, k, for 95 % expanded uncertainty intervals is based on a t multiplier with the appropriate associated degrees of freedom.

Information Values: Additional measurements and data were obtained to further characterize the material and are provided as information values. NIST information values are considered to be of interest to the RM user, but insufficient information is available to assess adequately the uncertainty associated with the values or a limited number of analyses were performed. Information values and associated measurement uncertainties for chemical and electrochemical properties unrelated to particle size are presented in Table 2. An optical absorbance spectrum is provided in Figure 1. Material sterility was assessed. Electron microscopy images are provided in Figure 3. Particle size histograms are provided in Figures 4 through 7. Information values cannot be used to establish metrological traceability.

Table 2. Information Value Mean and Measurement Uncertainty[a]
Chemical and Electrochemical Properties

Measurement	Value
Au mass fraction (μg g^{-1}) [b]	51.56 ± 0.23
Cl^- ion mass fraction (μg g^{-1}) [c]	35.0 ± 4.6
citrate ion mass fraction (μg g^{-1}) [c]	1.7 ± 0.4
Na mass fraction (μg g^{-1}) [d]	95
pH [e]	7.19 ± 0.33
electrolytic conductivity, κ (μS cm^{-1}) [f]	417.9 ± 7.2
zeta potential (mV) [g]	--

[a] For pH, conductivity and Au mass fraction, the expanded uncertainty (95 % confidence interval) is calculated according to the ISO/JCGM Guide [2]. Other reported uncertainties are two times the standard deviation of replicate measurements.

[b] Au bound into nanoparticles was determined from separate measurements of total Au and Au dissolved in the solution matrix. Both measurements were made using inductively-coupled plasma optical emission spectrometry (ICP-OES). Total Au was measured after digestion of the particles with a mixture of nitric and hydrochloric acids. Solution matrix Au was measured after removal of Au particles by ultracentrifugation and was undetectable at the 3σ detection limit corresponding to 0.07 μg g^{-1} in the undiluted supernatant. The Au mass fraction in the matrix was estimated as 0.5 time the 3σ limit and subtracted from the total Au mass fraction to obtain the reported value for the bound Au mass fraction.

[c] Levels of Cl^- and citrate ($C_3H_5O(COO)_3^{3-}$) ions were determined in native suspensions by ion chromatography with a conductivity detector. Chloride and citrate ions were identified based on the retention times of reference standards. Chloride levels in the water blank used to prepare calibrants were insignificant for this analysis. Citrate was not detectable in the water blank. The levels of Cl^- and citrate ions appear to increase slightly as a consequence of centrifugation, but this effect has not been quantified. Citrate bound to Au particles will not be detected by this approach.

[d] Na mass fraction was determined in the native suspension using inductively-coupled plasma optical emission spectrometry (ICP-OES). Matrix effects and other factors that may affect metrological validity are unaccounted for in this case. Additionally, Na may leach into solution from the inner surfaces of borosilicate glass ampoules. Ongoing studies at NIST have shown that Na mass fractions in excess of 10 μg g^{-1} may result under acidic conditions. The proportion of the observed Na mass fraction that is attributable to leaching is unknown. Furthermore, changes in the Na mass fraction over time are unpredictable.

[e] The pH was determined at 25.0 °C using a combination electrode with ceramic reference junction and a 2 point calibration referred to SRM 186g (pH 6.864) and SRM 187e (pH 9.186).

[f] Electrolytic conductivity was determined at 25.0 °C at 1 kHz using a dip cell with nominal cell constant of 0.1 cm^{-1}. The cell constant was determined using SRM 3191 (nominal κ, 100 μS cm^{-1}) and SRM 3192 (nominal κ, 500 μS cm^{-1}).

[g] Possibly due to the small particle size of RM 8011, zeta potential results obtained by Doppler velocimetry were of insufficient quality and precision to permit reporting. Results were sufficient only to confirm the Au particles carry a net negative charge.

Optical Absorbance: Optical absorbance spectra were obtained using a double-beam spectrophotometer on native suspensions. Measurements were performed using matched quartz cuvettes (10 mm path length) against a filtered deionized water reference. Scan conditions were: slit width, 1 nm; scan rate, 240 nm min^{-1}. The coefficient of variation determined near the plasmonic peak center was 0.2 % for spectra obtained from 6 randomly selected ampoules. A representative spectrum is presented in Figure 1.

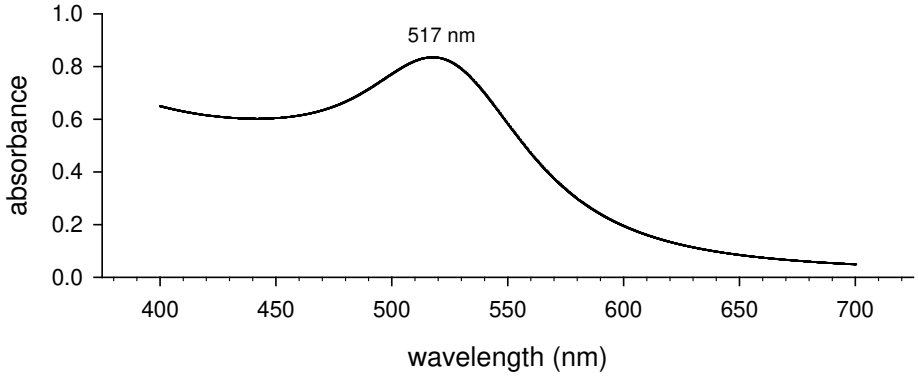

Figure 1. Representative optical absorbance spectrum for the native suspension, centered on the surface plasmon resonance peak for Au. Wavelength at peak maximum is indicated.

Sterility and Endotoxin Assessment[1]**:** Sterility was tested by plating RM 8011 on standard Luria-Bertani (LB) culture plates. No colony formation was observed after two days of incubation on LB plates for samples taken from ampoules before or after sterilization with gamma radiation. Endotoxin levels were not assessed due to interference from Au particles in the colorimetric detection used for the assay.[2]

Electron Microscopy Imaging: Representative micrographs are presented in Figure 2.

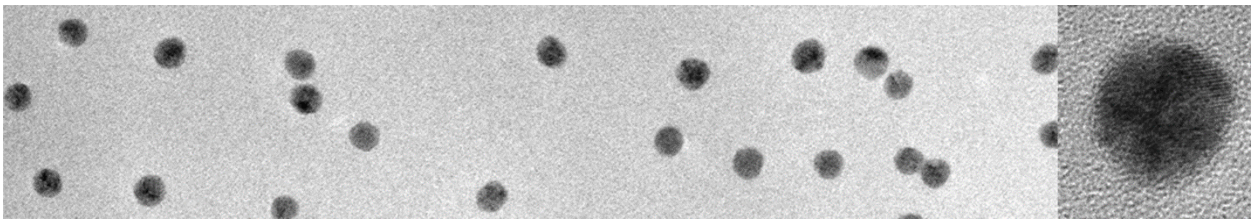

Figure 2. TEM micrographs. On left: image of Au particles sampled from a single representative TEM image. On right: high magnification TEM image revealing internal structure and faceting of a single Au particle.

Size Distribution Histograms: Histograms generated by AFM, SEM, TEM and ES-DMA (single particle analysis methods) are shown below in Figures 3, 4, 5 and 6, respectively. Histograms reflect the primary particle size distribution and do not necessarily reflect the presence of aggregates or potential artifacts. Binning was performed at a resolution of approximately 5 bin nm^{-1}.

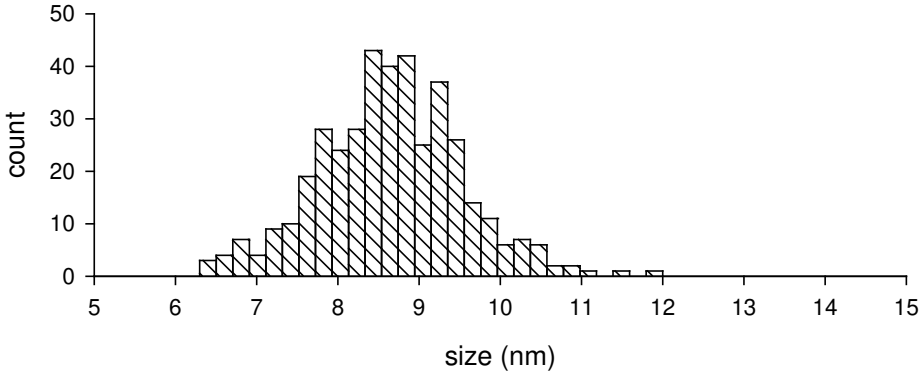

Figure 3. Particle size histogram generated by AFM analysis.

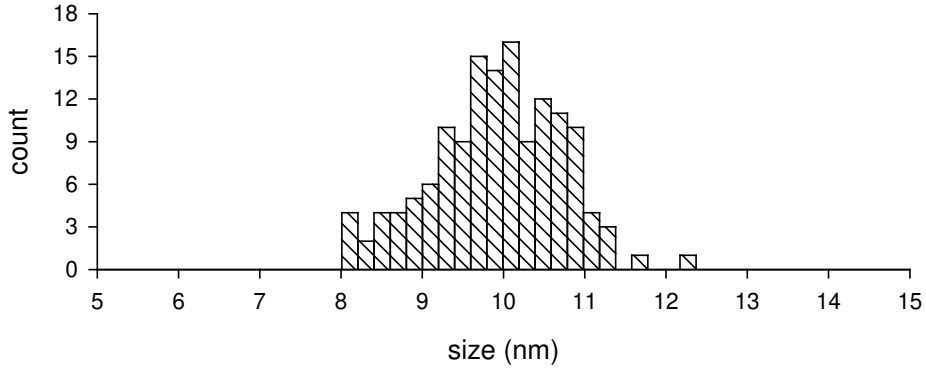

Figure 4. Particle size histogram generated by SEM analysis.

[1] Certain commercial equipment, instruments or materials are identified in this report to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

[2] The limulus amoebocyte lysate (LAL) assay was used to detect and measure bacterial endotoxin. Due to interference at the wavelength (405 nm) used to quantify the results of the underlying enzymatic reaction, Au particles must be removed from the solution by centrifugation prior to analysis. RM 8011 could not be adequately clarified by centrifugation at 20,000 g. Assays carried out on Au suspensions processed in an identical manner and from the same commercial source, but with larger particle sizes, resulted in no detectable endotoxin at a level of 2 pg mL^{-1}.

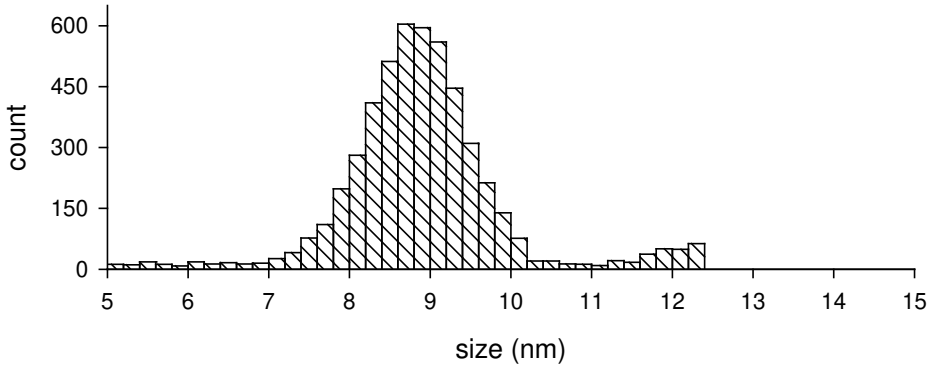

Figure 5. Particle size histogram generated by TEM analysis.

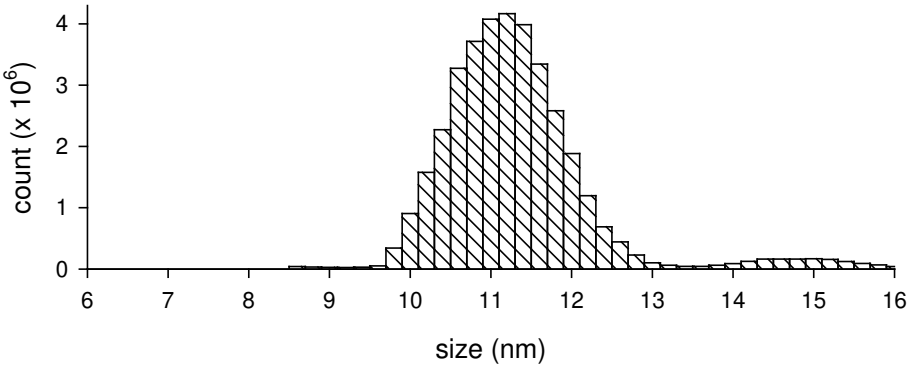

Figure 6. Particle size histogram generated by ES-DMA analysis. Bins containing counts attributable to salt particles have been removed.

NOTICE AND WARNING TO USERS

Caution: Ampoule contents **should not be allowed to freeze**, as this will permanently compromise the integrity of the material and invalidate reference values. A color change from red-pink to purple or clear indicates that the RM has been compromised. Occasionally, a visible black speck will be observed in an ampoule containing an otherwise translucent red-pink (i.e., normal) solution; this does **not** indicate the sample has been compromised; the specks settle rapidly and can easily be separated from the test material.

Warning: Not for clinical use or human consumption.

INSTRUCTIONS FOR HANDLING, STORAGE, AND USE

Handling and Storage: Until required for use, the RM should be **stored at room temperature** in its original ampoule and package and protected from intense direct light or ultraviolet radiation. Refrigeration is not necessary and is discouraged. Ampoules are best stored long term in a horizontal position.

Use: Prior to opening, the glass ampoule containing the RM should be gently inverted several times to insure homogeneity and resuspension of any settled particles. Liquid retained in the upper portion of the ampoule (the nipple), can be dislodged by gently flicking the nipple with forefinger while tilting the ampoule. The ampoule is pre-scored and should be opened by applying moderate pressure with one's thumb to snap off the nipple. It is recommended that the contents of an ampoule be used the same day as opened. Clean laboratory sealing film can be applied to seal a previously opened ampoule for short term storage. If it is necessary to use an ampoule over two or more days, then certain precautions should be taken: opening the ampoule in a clean bench (HEPA filtered) environment using sterile procedures (ethanol rinse), and sealing with ethanol-rinsed laboratory sealing film (optionally, one can transfer the suspension to a clean, sterile plastic or glass vial with a sealing cap), should prolong the useful life of opened ampoules for up to 7 days. Viability after longer term storage cannot be guaranteed but may be possible if these additional precautions are followed.

PREPARATION AND ANALYSIS

Material Source and Processing: The material used to produce RM 8011 was purchased from BB International of Cardiff, UK. A colloidal Au suspension was prepared to NIST specifications using the citrate-reduction method in a single 8 L batch at their manufacturing facility in the UK. The suspension was shipped in 1 L polycarbonate bottles and recombined at NIST in a sterile protein-free 10 L borosilicate glass flask. Recombination was performed in a HEPA-filtered clean bench using sterile procedures. The suspension was subsequently flame-sealed into Wheaton 5 mL pre-scored USP Type I glass ampoules using an automated process. Prior to use, the ampoules were cleaned with high pressure deionized water and autoclaved, then flushed with argon gas prior to and during filling. The sealed ampoules containing the Au suspension were sterilized with cobalt-60 gamma radiation to a minimum dose of 31.9 kGy by Neutron Products Inc. of Dickerson, MD.

Heterogeneity Assessment: During the filling process, ampoules were stored in boxes numbered 1 through 11, with box number corresponding to fill order. Heterogeneity testing was performed using measurements of optical density (OD) at 520 nm, hydrodynamic size, and relative Au mass fraction. Measurements of OD and hydrodynamic size (determined by dynamic light scattering), were performed on native solutions. For these measurements, two samples were extracted from each of 11 randomly selected ampoules (one from each box), for a total of 22 samples for each method. Both likelihood ratio [3] (to check if a model that ignores the ampoule effect and a model with the ampoule effect are similar) and ANOVA [3] tests conclude that ampoules are homogeneous for hydrodynamic size. Likelihood ratio indicates an apparent relationship between box number and OD (and thus statistical evidence exists to claim the models are not similar), but ANOVA indicates there is no significant ampoule effect. It is concluded that the engineering significance of the difference is negligible.

Au content was evaluated using inductively-coupled plasma optical emission spectrometry performed on 4 samples extracted gravimetrically from each of 11 randomly selected ampoules (one from each box). Analysis followed addition of an internal standard, acid digestion, and dilution with high-purity water. Relative Au mass fraction was calculated as relative instrument sensitivity values. ANOVA suggests that ampoules are different for a level of confidence of 95 %. It should be noted that the magnitudes of the observed heterogeneities are probably negligible in relation to the intended use of this material, since the largest and smallest ICP-OES relative sensitivity values differ by approximately 0.4 %. Likelihood ratio concludes that the ampoules are homogeneous.

Value Assignment and Uncertainty Analysis: Analyses to establish reference values were conducted at NIST using best practices as determined independently for each measurement method. Analyses were performed on replicate (typically two) subsamples drawn from randomly selected (typically four) ampoules of material; subsample sizes and methods were left to the discretion of the expert analyst. For AFM, the reference value is the mean of the measurement results, and the 95 % expanded uncertainty interval was obtained by applying the BoB (Type B on Biased) approach [4], using a rectangular distribution. For SEM, the reference value is the mean of the measurement results and the uncertainty level is based on a confidence interval approach [2], with an expanded uncertainty calculated as $U = ku_c$, where the combined uncertainty (u_c) is calculated as the estimated standard deviation of the mean and the coverage factor (k) is the expansion factor of 2 based on the Student's t multiplier associated with a level of confidence of 95 %. For TEM and ES-DMA, reference values were calculated from the ampoule means and the uncertainty level is based on a prediction interval approach [5], where the combined uncertainty is calculated as the standard deviation of the ampoule means multiplied by $\sqrt{1+1/N}$ (N is the number of ampoules analyzed) and the coverage factor is based on a t multiplier with N-1 degrees of freedom, for a 95 % expanded uncertainty interval.

METHODS FOR REFERENCE VALUE MEASUREMENTS

Atomic Force Microscopy (AFM): AFM probes the surface forces between a cantilever tip and the sample deposited on a flat substrate. The tip is rastered over the analysis area producing a 3D topographic image. Height measurements can be obtained with sub-nanometer precision. A Veeco Multimode AFM was used for measurements. Height measurements were calibrated using a silicon step-height transfer artifact (SH70-C19-R19) with a value of 68.9 nm ± 0.7 nm (NIST Calibrated-AFM, Precision Engineering Division) following the prescribed procedure for calibration. Intermediate contact ("tapping") mode was used with a Veeco RTESP phosphorus (n) doped silicon cantilever for imaging (resonance frequency 300 kHz, spring constant 40 N m^{-1}). Atomically flat polycrystalline Au on mica was used as a substrate in order to provide a consistent baseline for size measurements with minimal interference from surface roughness.

To prepare samples for analysis, approximately 1 mL aliquots of native suspension from 2 randomly selected ampoules were placed into 1.5 mL microtubes and centrifuged at 14 krpm for 20 min. A portion of the supernatant from each microtube was then removed and replaced with deionized water to obtain an 8-fold dilution of the native suspension. No change in stability of the suspension was observed during this process. A droplet of each diluted suspension was then placed on the Au substrate and dried at 70 °C. The maximum height with reference to the baseline substrate was

recorded as the size (diameter) of the Au particle. Images were collected from different areas of the deposited substrate. Height profiles representing 300 particles from one ampoule and 100 particles from the second ampoule were individually analyzed to acquire the size distribution and mean.

Scanning Electron Microscopy (SEM): In SEM the sample is imaged using low-energy secondary electrons in a process that employs a raster-scanned primary beam. An FEI Helios Dual-Beam SEM was used for imaging, with the following conditions: 15 keV accelerating voltage, 86 pA beam current, 30 µs beam dwell time for each image pixel, and 3.5 mm sample working distance. Image contrast and brightness were set so that a good balance between detail and distinction from background was achieved. For scale calibrations of X and Y directions a VLSI Standards NanoLattice sample was used. This artifact was calibrated on NIST's Calibrated Atomic Force Microscope (C-AFM) by R.G. Dixson of the NIST Precision Engineering Division, who determined a pitch value of 99.98 nm with an uncertainty of 1.5 nm ($k = 2$). Samples were imaged at 500 k× magnification. A digital capture resolution of (2048 × 1886) pixels was used for all images. Under these conditions, a nominal 10 nm particle will yield an area of roughly (35 × 35) pixels.

The software package ImageJ v1.37 (available from the National Institutes of Health: http://rsb.info.nih.gov/ij/) was used for image processing and data analysis. The Otsu threshold algorithm was implemented to produce a binary image in which the particles are white and the background is black. The outlines of particles as traced by ImageJ were used to check the quality of the particle separation from their background in order to discriminate between single particles and aggregates. The area data for each numbered particle as obtained from ImageJ were first converted to an effective spherical diameter value in pixel units, which was then converted to length units (nm) based on the pitch calibration. A total of 140 particles were analyzed on samples prepared from 2 randomly selected ampoules.

Substrates were prepared by placing a drop of aminopropyldimethylethoxysilane (ADMES) on a clean 5 mm × 5 mm Si substrate cleaved from a 100 mm diameter wafer. The untreated wafer supports a thin, native oxide layer. The ADMES was allowed to react for 2 h to 6 h, after which excess silane was rinsed off with isopropanol followed by deionized water. For analysis, the Au particles were then deposited onto the derivatized substrate by contacting with a droplet of native suspension for a period of 1 h to 2 h. The deposited substrate was then rinsed with isopropanol followed by deionized water, and dried by gently blowing with filtered dry nitrogen prior to analysis. Samples were analyzed as deposited; a conductive coating was not required.

Transmission Electron Microscopy (TEM): TEM measures the projected image of particles deposited onto an electron-transparent substrate. Internal structure, as well as surface morphology, may contribute to the image appearance. A Philips EM400T TEM, operating at 120 kV and equipped with an Olympus Cantega bottom mount CCD camera, was used for measurement of deposited samples. Frames were captured at an exposure time of 2 s. The magnification of the microscope/camera system was calibrated using negatively stained catalase crystals and analyzed by the CRISP software package (http://www.calidris-em.com/). TEM images were analyzed in the IgorPro (http://www.wavemetrics.com/) software package using custom macros written by B.M. Vogel of the NIST Polymers Division. Particle size was determined by measuring the contiguous area of pixels that fall within the threshold set for a particular micrograph. This area was then used to determine the equivalent diameter assuming a spherical particle. Therefore, the average particle size does not consider any pronounced faceting. The circularity value, defined as $P^2/4\pi A$ (where P is the particle perimeter and A is its area), approaches 1 for an ideal circle. Particles with circularities of 6 or higher were not counted, in order to minimize aggregate/artifact inclusion in the size analysis.

The substrate consisted of a 3 mm Cu grid with a 10 nm continuous film of silicon monoxide that was functionalized with aminopropydimethylethoxysilane (ADMES). The substrate was prepared by contacting a commercial grid with about 20 µL of ADMES while sealed in a glass vial to trap vapor and prevent evaporation. After 1 h, the grids were removed and dip-washed in ethanol and allowed to dry. To prepare a sample for analysis, one droplet (roughly 8 µL) of native Au suspension was placed on a functionalized grid presented on a stud suspended above a reservoir containing water. A cover was placed over the assembly to prevent evaporation of the Au suspension. After 1 h the grids were dip-washed in distilled water and then ethanol to remove any remaining suspension. The grids were then dried at room temperature prior to measurement. A total of 5098 particles were analyzed on grids prepared from 4 randomly selected ampoules.

Electrospray – Differential Mobility Analysis (ES-DMA): In ES-DMA the liquid suspension is first conveyed into the gas phase using electrospray ionization. The resulting droplets pass through a neutralizing chamber where collisions with charged ions reduce the charge to a modified Boltzmann distribution [6]. Consequently, most of the positively charged particles left after the droplets evaporate possess a single net charge. As they dry, residual salts or other nonvolatile impurities encrust the surface. Within the analysis chamber charged particles are attracted to a negatively biased center electrode, while being dragged along by a carrier gas. Particles for which the electrical force balances the drag force pass through a collection slit, after which a condensation particle counter enumerates the

number of particles passing through the detector per cubic centimeter of gas flow. Stepping through the voltage yields a particle size distribution. The experimental system used in this study consisted of an electrospray aerosol generator (Model 3480, TSI Inc.), a differential mobility analyzer (Model 3080n, TSI Inc.) and a condensation particle counter (Model 3025, TSI Inc.). The following conditions were used: capillary diameter, nominally 25 μm; electrospray voltage, 1.67 kV to 2.78 kV; CO_2 pressure and flow rate, 6.89×10^4 Pa and 0.2 L min^{-1}; air pressure and flow rate, 2.55×10^4 Pa and 1.0 L min^{-1}; sheath/carrier gas flow rate, 30 L min^{-1}; flow entering the particle counter (supplemented by filtered air), 1.5 L min^{-1}. The baseline cut off value was set at 30 counts to ensure clear separation between peaks and to account for baseline noise.

Conversion of DMA voltages to equivalent diameters and generation of the particle size distribution were achieved using equations and parameters specified by the commercial vendor of the ES-DMA instrumentation. To account for the thickness of any nonvolatile salts encrusted on the surface of the particles, the mode diameter of the salt peak was determined and subtracted from the subsequent particle sizes, after which the number average diameter was calculated for each sample.

From each of 4 randomly selected ampoules, 900 μL of native suspension was transferred to low-binding microfuge tubes and centrifuged for 40 min at 13.2 krpm. The clear supernatant was removed, leaving 20 μL to 30 μL of material to which was added 500 μL of 2 mmol L^{-1} ammonium acetate solution at pH 8. A vortex mixer was used to re-homogenize samples, which were then subjected to ES-DMA.

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Users of this RM should ensure that the Report of Investigation in their possession is current. This can be accomplished by contacting the SRM Program: telephone (301) 975-2200; fax (301) 948-3730; e-mail srminfo@nist.gov; or via the Internet at https://www.nist.gov/srm.