

# **Laser Detection of Nanoparticles In the Environment**

## **APM 31**

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## FOREWORD

The United States Environmental Protection Agency (EPA) is charged by Congress to protect the nation's natural resources. Under the mandate of national environmental laws, the EPA strives to formulate and implement actions leading to a compatible balance between human activities and the ability of natural systems to support and nurture life. To meet this mandate, the EPA's Office of Research and Development (ORD) provides data and scientific support that can be used to solve environmental problems, build the scientific knowledge base needed to manage ecological resources wisely, understand how pollutants affect public health, and prevent or reduce environmental risks.

The National Exposure Research Laboratory (NERL) is the Agency's center for investigation of technical and management approaches for identifying and quantifying exposures to human health and the environment. Goals of the laboratory's research program are to: 1) develop and evaluate methods and technologies for characterizing and monitoring air, soil, and water; 2) support regulatory and policy decisions; and 3) provide the scientific support needed to ensure effective implementation of environmental regulations and strategies.

This report presents the experimentation, results and findings to date, relating to methods of characterization of sample solutions containing nanoparticles of silver and polystyrene, using *nanoparticle tracking analysis* (NTA). Also, this report is directed at confirming the efficacy of NTA and comparing it to other methods to determine nanoparticle sizes and size distributions in water samples. Included in the report are recommendations for additional work to address issues identified herein.

NTA technology allowed for the direct visualization nanoparticles and has a unique software tracking system to determine particle sizes. The goal of this research was to confirm the applicability of NTA as a screening method for nanoparticles in environmental water samples. An additional potential goal would be to support site remediation programs (e.g., Department of Defense and EPA Office of Solid Waste and Emergency Response) and other applications to identify possible engineered nanomaterials contamination and to track the transport and fate of these nano-contaminants.

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## EXECUTIVE SUMMARY

This report covers efforts to investigate a relatively new technology, *nanoparticle tracking analysis* (NTA) for use in identifying nanoparticles. NTA is a laser-based instrument capable of real-time tracking of nanoparticles in solution. NTA was evaluated as a stand-alone screening method to identify and calculate particle size distributions of metal-based, engineered nanomaterials (ENMs) *in situ* and in laboratory synthesized environmental water samples. Based on the information considered here, overall NTA sensitivity appeared to be good for particle sizes in the 20 - 40 nm range, and larger sizes, including the ability to follow the real-time formation of aggregates. Reliable data was more difficult to obtain for particle sizes below the 20 nm size range. Benefits of the NTA technology were that the instrumentation allowed for visualization of nanoscale particles in liquids on an individual basis, provided nanoparticle size and distribution data for each sample, and was capable of measuring large numbers of individual particles in substantially less time than required by microscopy techniques, i.e., transmission electron microscopy (TEM), scanning electron microscopy (SEM), atomic force microscopy (AFM). Also included in this report are comparisons of NTA with a number of other nanoparticle characterization technologies including ultraviolet-visible spectroscopy (UV-Vis), dynamic light scattering (DLS), TEM, and ultra small angle X-ray scattering (USAXS).

In addition to size distribution, there are several other aspects of nanoparticle tracking to be considered. These include sensitivity/selectivity to discriminate between metal-bearing ENMs and other background naturally occurring nanomaterials in water samples, influence of particle surface coatings and particle tendencies to agglomerate, among others. These issues are discussed and if NTA is used as a screening technique it is suggested that a complementary nanodetection method, e.g., SEM, TEM, AFM, be considered as a part of the screening process.

## LIST OF ACRONYMS AND ABBREVIATIONS

APS	Advanced Photon Source
AFM	Atomic Force Microscopy
AF4	Asymmetric Field Flow Fractionation
AgNP	silver nanoparticle
DI	deionized
DLS	Dynamic Light Scattering
ECs	emerging contaminants
EDX	energy dispersive X-ray
ENM	engineered nanomaterials
EPA	Environmental Protection Agency
ESD	Environmental Sciences Division
eV	electron volts
g	gram
HEASD	Human Exposure and Atmospheric Sciences Division
HDC	hydrodynamic chromatography
h	hour
ICP-MS	Inductively coupled plasma-mass spectrometer
k	1000
kg	kilogram
LIBD	Laser-Induced Breakdown Detection
L	liter
mg	milligram
MHRW	moderately hard reconstituted water
mL	milliliter
MS	mass spectrometer
ng	nanogram
nm	nanometer
NERL	National Exposure Research Laboratory
NIST	National Institute of Standards and Technology
NIST-NCL	National Institute of Standards and Technology-Nanotechnology Characterization Laboratory
NOM	natural organic matter
NP	nanoparticle
NRC	National Research Council
NTA	Nanoparticle Tracking Analysis
ORD	Office of Research and Development
OSWER	Office of Solid Waste and Emergency Response
PI	polydispersity
ppb	part-per-billion
PPE	personal protective equipment
PPCP	pharmaceutical and personal care product
PVDF	polyvinylidene
PVP	polyvinylpyrrolidone

## LIST OF ACRONYMS AND ABBREVIATIONS (continued)

s	second
SEC	Size exclusion chromatography
SEM	Scanning Electron Microscope
SP	single particle
SPR	Surface Plasmon Resonance
TEM	Transmission Electron Microscopy
μL	microliter
UNLV	University of Nevada – Las Vegas
US	United States
USAXS	Ultra Small Angle X-Ray Scattering
UV	ultraviolet
UV-Vis	Ultraviolet Visible Spectroscopy
V	volts
WWTP	wastewater treatment plant
Z-avg	nanoparticle equivalent hydrodynamic diameter

## 1.0 INTRODUCTION

Naturally occurring nanoparticles have always been around, created either by the weathering of minerals (e.g., gold, silver, copper), forces of nature (e.g., forest fires, volcanoes), or incidentally (e.g., emissions from combustion sources). However, anthropogenically engineered nanomaterials are recent inventions (Owen et al. 2007; Lubick 2008; Farré et al. 2009). “Nanotechnology” involves the manipulation and engineering of chemicals for structures in the size range of 1 to 100 nm in at least one direction (one nanometer is  $10^{-9}$  meters). These nanomaterials can be considered as emerging contaminants (ECs), and are engineered from nanometallic (e.g., silver, gold, iron) and nanocarbon (e.g., fullerenes) materials that are sized between 1 nm and 100 nm. The Woodrow Wilson Institute, since 2006, has kept an on-line database of the number of consumer nanomaterials products currently being offered on the market (WWIC 2011). The number of nanomaterial-containing products has grown substantially from 212 products listed in 2006 to 1317 products as of September 2011 (WWIC 2011).

It can be difficult to obtain information on total production volumes of nanoparticles (much of which would be potentially available to the environment), because the production information is often proprietary. Publicly available sources (which vary considerably) provide estimates of nanoparticle  $\text{TiO}_2$  production exceeding 50,000 tons per year in 2010 in the United States (US) (BusinessWire 2011). Worldwide production of silver nanoparticles is much lower, but has been estimated at approximately 500 metric tons per year in 2008 (Mueller et al. 2008). The current trend seems to be for increased production of all nanomaterials.

It can be expected that a large portion of nanomaterials will eventually find their way into the environment, especially through wastewater treatment plants (WWTPs) (USEPA 2010). This can be either through WWTP effluent, or via land-applied biosolids (the solid material leftover during WWTP processes)(Geranio et al. 2009). As of 2002, WWTP biosolids usage, in the US, was on the order of 3 million dry tons per year as amendments for soil treatment (NRC 2002).

However, the effects of nanoparticles on humans and the environment are not yet fully understood, and there are mixed views on the risks. While there is evidence of nanosilver toxicity to aquatic organisms found in the environment (Kennedy et al. 2010), there are currently few known harmful effects on humans, and some possibly beneficial effects, from exposure to certain nanoparticles. For example, nanosilver is presently used in medical applications as a disinfectant in wound dressings and in catheters and breathing tubes to reduce incidents of infections (NCCAM 2010). Moreover, in considering these risks, it is necessary to go beyond just the particulate form of silver because, when released to the environment, the nanoparticles can be converted to the ionic form of silver which can be more toxic than the particulate form (Liu et al. 2010). Even less is known about human and environmental effects from other nanomaterials (e.g., nanotitanium, nanoaluminum, nanocarbon) that are gaining wider usage.

Given anticipated increases in total nanomaterials usage, the widespread and varied applications for these materials, and a current lack of consensus standards and scientific opinion regarding associated human and environmental risks, it is becoming ever more important to be able to characterize nanomaterials in the environment. The development of a relatively quick and reliable screening test(s) to correctly identify and characterize nanomaterials in various environmental media would be beneficial. The ability to obtain such data more efficiently is expected to aid researchers in focusing their efforts in evaluating the potential exposures and risks posed by nanomaterials. To provide that ability was the objective for this effort on laser detection of nanoparticles in the environment.

## **2.0 RESEARCH GOALS**

The primary goal of this research effort was to evaluate the effectiveness and efficiency of a relatively new instrumentation technology, nanoparticle tracking analysis (NTA). The NTA technique (NanoSight, Ltd LM-20) uses a laser detection method to highlight nanoparticles, which can then be viewed using a conventional optical microscope. NTA uses sophisticated particle tracking software, based on Brownian motion and the Stokes-Einstein equation, to calculate the sizes of the particles. NTA was evaluated to see if it could provide relative concentrations and size distributions of metal-based nanoscale particles in liquid suspensions on an individual basis.

The secondary goal of this research effort was intended to provide data for suitability comparisons between the NTA technology and several other available detection methods (i.e., DLS, TEM, UV-Vis, and USAXS) which are detailed later in the report (Section 4.4). This phase of research consisted of a lab-based study to initially establish the suitability of the technology as a stand-alone method, or complementary to other engineered nanomaterial (ENM) detection methods, to screen laboratory synthesized environmental waters for ENM particles and potential ENM contamination.

## **3.0 NANOPARTICLE DETECTION METHODS**

There are many techniques that have been reported in the literature that are used to detect and size nanoparticles. Some are microscopy-based, like TEM, SEM and AFM (Boetz et al. 2004; Buhr E et al. 2009; Boyd et al. 2011; Klein et al. 2011). Others are based on light and absorbance or emission of light through back-scattering of the light and use of software to track the Brownian motion of the light using the Stokes-Einstein equation, such as DLS and NTA (Filipe et al. 2010; Boyd et al. 2011). Other techniques are based on size exclusion with a detector at the end, for example, size exclusion chromatography (SEC), gel electrophoresis, asymmetrical flow field-flow fractionation (AF4), ultracentrifugation, and hydrodynamic

chromatography (HDC) [coupled with inductively coupled plasma–mass spectrometry (ICP-MS)](Bootz et al. 2004; Brown et al. 2006; Messaud et al. 2009; Pergantis et al. 2011)

The research reported herein includes comparisons of NTA with several of the characterization technologies: UV-Vis, DLS, TEM, and USAXS.

**3.1 General Discussion.** The NTA, UV-Vis and TEM research was conducted by EPA: Dr. Manomita Patra, a National Research Council (NRC) post-doctoral research fellow [Mrs. Tammy Jones-Lepp (National Exposure Research Laboratory-Environmental Sciences Division, NERL-ESD) was the EPA-NRC advisor for Dr. Patra] collected the NTA and UV-Vis data, and the TEM data was collected by Dr. Kim Rogers (NERL-Human Exposure Atmospheric Sciences Division, HEASD). The DLS, AFM, USAXS data were collected by Dr. Robert MacCuspie, Andrew Allen, Matthew Martin, and Vincent A. Hackley at National Institute Standards and Technology (NIST), Gaithersburg, MD.

**3.2 Assessment Criteria.** To provide a suitable evaluation of available methods for characterizing nanoparticles in the environment, a multi-step evaluation was used. This evaluation included examining the following criteria:

1. Ability to detect particles in the 1 – 100 nm size range.
2. Allow visualization and counting of nanoparticles on an individual basis.
3. Detection of particles in pure water, natural organic material water, and solvents.
4. Detection in concentrations in the  $10^6$  to  $10^9$  particles per mL range.
5. Sensitivity to differentiate between ENM and natural nanoparticles.

**3.2 Initial Candidate List.** Only nanosilver (AgNP), and polystyrene beads, with particle sizes of 1-100 nm, were evaluated. The intent was to identify a screening test with wide applicability and the comparisons discussed herein included both laser and non-laser detection methods. Following is a list of each method evaluated and primary equipment used.

<b><u>Method</u></b>	<b><u>Equipment and Laboratory testing the equipment</u></b>
UV-Vis Spectroscopy	Perkin Elmer Lambda 750 spectrophotometer Hewlett Packard Model 8453 (NERL-EPA) *
Dynamic Light Scattering	Malvern Instruments Zetasizer Nano (NIST) <sup>†</sup>
Nanoparticle Tracking Analysis	NanoSight LM20 (NERL-EPA)
Transmission Electron Microscopy	FEI Technai TEM (NERL-EPA using UNLV <sup>††</sup> equipment)
Atomic Force Microscopy	Dimension 3100 AFM (NIST)
Ultra Small Angle X-Ray Scattering	Advanced Photon Source (APS) (NIST)

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## 4.0 EXPERIMENTAL MATERIALS AND METHODS

**4.1 AgNP Materials.** AgNPs were selected to provide as broad of a range as possible of sizes, capping agents, and powder/suspension states. The vendor-reported nominal diameters were relied upon as accurate and were used as the diameters for AgNPs. Two separate batches of AgNPs were obtained for use at NERL-Las Vegas and NIST. Figure 1 illustrates a flow chart of samples received and subsequent preparation techniques.

Citrate-capped AgNPs were obtained from several sources, including (10, 20, and 100) nm from the NanoXact product line (Nanocomposix, San Diego, CA, USA), (20, 40, 60, and 80) nm from the unconjugated silver colloids BBI product line (Ted Pella, Redding, CA, USA), and 20 nm from Microspheres Nanospheres (Cold Spring, NY).

Starch-capped (10 to 15) nm AgNPs were obtained from Strem Chemicals (Newburyport, MA, USA).

Polyvinylpyrrolidone (PVP)-capped AgNPs (10 and 50) nm were obtained from the NanoXact product line (Nanocomposix, nominal, San Diego, CA, USA) and (10, (30 to 50), and 50) nm from NanoAmor (Houston, TX, USA).

Oleic acid capped (30 to 50) nm AgNPs were obtained as powders from NanoAmor (Houston, TX, USA). No capping agent specified AgNPs (1 to 10) nm were obtained from Vive Nano (Toronto, Canada).

**4.2 Dispersion Preparation.** AgNPs were analyzed as-received, diluted with deionized (DI) water, diluted with EPA moderately hard reconstituted water (MHRW) (Hackley et al. 2007), or MHRW plus Suwannee River Fulvic Acid Standard I or Suwannee River Humic Acid Standard II (1S101F and 2S101H, respectively, International Humic Substances Society, St. Paul, MN, USA) at a natural organic material (NOM) concentration of  $10 \mu\text{g mL}^{-1}$ . AgNPs were added to diluents, shaken by hand for 2 s, then allowed to stand for at least 1 h before measurements began. Stock dispersions of AgNPs received as powders were prepared by adding 1.0 mg of AgNP powder to 1.000 mL of DI water and sonicating in a bath sonicator for 10 min.

Before some measurements of the reconstituted AgNPs were made, the suspensions were passed through a  $0.45 \mu\text{m}$  polyvinylidene fluoride (PVDF) syringe filter. DI water was produced using  $18.2 \text{ M}\Omega\cdot\text{cm}$  water obtained from a Aqua Solutions (Jasper, GA, USA) Type I biological grade water purification system outfitted with an ultraviolet lamp to oxidize residual organics and a low relative molecular weight cut-off membrane, then passed through a  $0.1 \mu\text{m}$  PVDF syringe filter before use. All AgNPs were stored at  $4 \text{ }^\circ\text{C}$  in the dark in their original containers, and were characterized within 6 months of receipt from vendors.

### 4.3 Methods and Instrumentation

In this section only those instruments that were used at EPA-NERL-Las Vegas to evaluate alongside NTA will be discussed, i.e., NTA, UV-Vis and TEM.

**4.3.1 Nanoparticle Tracking Analysis (NTA).** Solutions were diluted to an approximate AgNP concentration range of  $10^9$  AgNPs per mL, and injected via a 1 mL disposable syringe into a Nanosight LM20 (Nanosight, Amesbury, United Kingdom) equipped with a 633 nm laser and low sensitivity detector. Rinsing with filtered DI water between samples cleaned the liquid cell. Fresh DI water, post-rinsing, was checked to ensure no cross-contamination of AgNPs occurred. The optics were adjusted by finding the immobile area of diffraction from the laser beam, or the so-called fingerprint area, and moving the liquid cell so that the volume closest to the fingerprint without interferences was the volume observed. Camera settings were adjusted empirically by maximizing the brightness of the AgNPs while minimizing any background light. Videos were collected for 30 s. Post-collection analysis parameters were adjusted empirically to maximize the number of particles correctly identified by the proprietary software (NTA version 2.0) while simultaneously minimizing the number of noisy pixels incorrectly identified.

As with most measurement techniques, incorrect use of the instrument can produce incorrect or misleading data. Thus, video analysis parameters including blur, detection threshold, gain, brightness, and number of completed tracks were adjusted independently, and systematically, to maximize the number of completed tracks obtained during a video analysis before reporting sizing results using those processing conditions.

**4.3.2 UV-Vis Spectroscopy.** UV-Vis spectra were collected on both a Perkin Elmer (Waltham, MA, USA) Lambda 750 and a Hewlett Packard (HP) 15 Model 8453 spectrophotometers, using UV-transparent disposable plastic semi-micro cuvettes (Brandtech, Inc., Essex, CT, USA), with a 1-cm path length, requiring 1 mL sample volumes. The Perkin Elmer spectrometer uses a split-beam configuration equipped with an 8 + 8 cell changer and water-jacketed 20 temperature control; measurements were performed at  $25.0 \pm 0.2^\circ$  C. The HP instrument has a single beam configuration, and measurements were taken at  $25^\circ$  C. Concentrated AgNP solutions were diluted such that the initial absorbance was approximately 1.0 at the surface plasmon resonance (SPR) absorbance peak wavelength ( $\lambda$  25 max).

**4.3.3 Transmission Electron Microscopy (TEM).** NIST-Nanotechnology Characterization Laboratory (NCL) Joint Assay Protocol PCC-7 was broadly followed (Bonevich et al. 2010). Briefly, amine-functionalized silicon TEM windows (Dune Sciences, Eugene, OR, USA) were immersed into an AgNP solution, incubated for 1.0 min, and then rinsed by immersion into filtered DI water. Images were acquired at 300 kV using a Technai TEM (FEI, Hillsboro, OR, US) with a 2k Gatan camera. The imaged structures were also analyzed by energy dispersive X-ray (EDX) spectroscopy for elemental composition using an

EDX detector. At least five locations on the TEM window (grid) were examined, and between 50 and 90 images were recorded. Image J software was used for image analysis, freely available on the internet (Rasband 1997-2011; Abramoff et al. 2004). Sizes were measured by using the line distance-measuring tool across the diameter of the AgNP, calibrated to the scale bar imprinted on the TEM.

## **5.0 RESULTS AND DISCUSSION**

**5.1 Uncertainties in Measurements.** The reported values from microscopy measurements are often the mean of all NPs measured with an uncertainty of one standard deviation about this mean. The uncertainty in this case reflects the width of the size distribution. Uncertainties in the mean of number-based particle sizing measurements for NTA can be reduced by increasing the number of particles sampled. This can be easily done by increasing the number of tracks recorded. On the other hand, techniques like DLS typically report the mean of several measurements performed under repeatability conditions with an uncertainty of one standard deviation about the mean of those measurements. Wherein, the uncertainty in this case represents the repeatability or precision of the measurements, and does not reflect the width of the distribution.

**5.1.1 NTA.** NTA mean and mode diameters were based on the number of AgNPs sized, or the number of completed tracks. Analysis of each sample yielded 100-500 individual track tracings that were used to determine mean, mode and relative standard deviation measurements. For this type of measurement, the variation about the mean is not an indication of the precision of multiple measurements of a single value, but is rather representative of the particle size distribution. For this reason, standard deviation values may be misleading and were not reported for this technique. Reported means for each particle type (Table 1) represent the average of five mean values from separate sample measurements. A useful indication of the relative size distribution measured using the NTA technique may be derived by comparison of mean and mode measurements (Table 2). The mode was typically associated with a peak (most commonly measured particle size to the nearest nm) close to the nominally reported particle diameter.

**5.1.2 TEM and AFM.** Error bars for TEM and AFM represent one standard deviation about the mean for all particles counted in a sample. Therefore, the error bars are representative of the width of the distribution and are not reflective of the uncertainty associated with determining the mean size.

**5.1.3 DLS.** For DLS, the mean of five replicate Z-avg measurements under repeatability conditions is reported with error bars of one standard deviation about the mean of the Z-avg values. The DLS error bars represent the repeatability of the DLS measurement (the ability to achieve the same measurement result consecutively) and do not indicate the width of the size distribution.

**5.1.4 USAXS.** For USAXS, an estimated measurement uncertainty of  $\pm 10\%$  is assigned to all values reported, based on previous experience measuring NIST AuNP reference materials RM8011, RM8012, and RM8013 (NIST 2008; NIST 2008; NIST 2008).

**5.2 Effects of sample matrix.** The conditions under which the materials are “initially” characterized can impact their reported size and size distributions, which in turn are used as the basis for interpretation of test results. To compare the strengths and weaknesses of each measurement technique, the AgNPs selected for the studies were analyzed as received or diluted with DI water (these are referred to as pristine conditions; Table 1). Alternatively, the samples were diluted into either MHRW, or MHRW with NOM standard I or II (MHRW+I or MHRW+II, respectively). The sizing results for each dispersion condition are summarized in Tables 1 and 2.

Figure 2 illustrates the differences between the reported nominal size and observed mean size for the citrate-capped AgNPs for the pristine materials (Figure 2a), and dilution into environmental media, MHRW (Figure 2b). The range of deviations from the nominal vs. measured line (dashed lines in Figure 2) illustrates the complexity of intercomparing results. Among the citrate- and PVP-capped AgNPs from various sources, certain trends were noted.

For example, for those particles that the nominal size was reported at  $< 20$  nm, the dry-state measurements provided by AFM and TEM showed average diameter values that differed by less than 10 nm from the nominal reported values in pristine conditions. However, the average hydrodynamic diameter measurements from NTA and DLS showed a difference of  $> 200$  nm, for those particles  $\leq 10$  nm. In pristine waters, in general AgNPs that were nominally  $< 20$  nm (Samples A, I, J, M, and N), mean diameter measurements by AFM and TEM ranged from 7.9 nm to 12.4 nm and 3.6 nm to 13.2 nm, respectively. By contrast, hydrodynamic diameters, as measured by DLS and NTA, ranged from 22.7 nm to 547 nm and 28.8 nm to 195 nm, respectively. The larger increases ( $> 300\%$  for DLS) for the hydrodynamic diameter measurements of these AgNPs suggest agglomeration of AgNPs. This would suggest an influence from surface coatings, a few large-sized outliers, and/or the presence of small agglomerates.

In pristine conditions, for most of the citrate- or PVP-capped AgNPs that were  $> 20$  nm DLS and NTA still followed a measurement method trend of measuring larger than the nominally reported sizes, but not as severe as at the smaller,  $< 20$  nm, particle sizes. TEM showed the most accuracy overall for all sizes of AgNPs, while AFM showed a trend of measuring less than the nominally reported size for those AgNPs that were  $> 60$  nm.

In solutions with MHRW, again we see that DLS and NTA trended toward measuring larger than the reported nominal size for those AgNPs that were < 10 nm. For those particles > 20 nm, NTA performed better than DLS in measuring closer to the nominal reported sizes, but both NTA and DLS still trended toward measuring larger than the nominal reported size, for all AgNPs. Again, suggesting an influence from surface coatings, a few large-sized outliers, and/or the presence of small agglomerates.

For those AgNPs in MHRW that were > 40 nm, the AFM measurement technique trended toward measuring less than the nominal reported value.

It is important to note that for multimodal distributions of AgNP sizes, as measured by DLS and NTA, the average size of all AgNPs observed in a number distribution may not be the most informative way to report the size. Identification of the mean or mode of each peak in the size distribution may be more appropriate (this will be discussed later in Sections 5.4 and 5.5). From a biological perspective, when attempting to assess the risk of a specific size ENM crossing certain biological barriers, or the available AgNP surface area per unit volume of solution, the small AgNPs in a sample may become critical to successful interpretation of data.

**5.3 Measurement Methods – Limitations.** All of the methods considered: NTA, UV-Vis, DLS, TEM, AFM and SAXS; can provide one or more measures of characterization of ENMs in liquid dispersions. However, no one method provides all of the capabilities that might be useful and/or desired. Therefore the following comments are only intended to provide insights into the relative merits of the methods considered for comparison to NTA in this report.

5.3.1 NTA provides data on nanoparticle size (hydrodynamic diameter) and distribution. This technique requires a somewhat narrow concentration range in order to be effective, and data for nanoparticles in sizes below 20 nm becomes questionable except for particles with a high refractive index (e.g. Au or Ag).

5.3.2 DLS provides data on size (hydrodynamic diameter) and distribution. Since it depends on Rayleigh scattering proportional to particle diameters; larger particle diameters will tend to dominate the intensity signal. DLS does not collect intensity (brightness) data and may not be able to distinguish between particles of comparable size but of different composition.

5.3.3 UV-Vis method is typically used to provide quantitative data on total concentration of nanoparticles, but only qualitative information regarding size and size distribution of nanoparticles.

5.3.4 TEM and AFM provide size and distribution data over all size ranges but are sensitive to sample preparation methods which can impact results. Both TEM and AFM tend to respond more to particle core sizes and may not fully reflect particle coatings and/or associated molecules that would increase the hydrodynamic diameter.

5.3.5 USAXS (similar to TEM) provides size and distribution data but is apparently not as effective in detecting small AgNPs (e.g. < 5 nm), especially in sample populations which also contain substantial numbers of large diameter NPs.

Since NTA and DLS both measure hydrodynamic diameters (reflecting coatings and aggregates), and AFM and TEM both measure diameters based on particle core size (not reflecting coatings or associated surface elements), these techniques are consequently complementary and one of each would be required to adequately characterize particle and aggregate size distributions. Consideration should also be given to the use of Scanning Electron Microscopy (SEM) in lieu of TEM. The SEM resolution is perhaps an order of magnitude less than TEM, but is still adequate for particles of about 1 nm in size. The SEM can image large bulk samples, has good depth of field for 3D image representations, and can be set up to produce adequate quality and resolution for environmental (“wet”) samples.

**5.4 Detection of ENMs under Pristine Conditions.** Figure 2a illustrates the range of mean sizes that could potentially be reported for AgNPs under pristine conditions, and Table 1 details the numerical sizing results.

The hydrodynamic diameters, as measured by DLS and NTA range from 22.7 nm to 547 nm and 18.2 nm to 195 nm, respectively. In contrast, the nominal sizes, reported by the vendors, ranged from 10 nm to 100 nm. The larger hydrodynamic diameters, sometimes greater > 300% than the nominal range, suggest the presence of AgNP agglomerates even in pristine water.

Agglomerates can be common for some of these particle types in solution in that clusters of nanos combine to be seen as single particles. Figure 4a, sample O shows that this agglomeration is probably what occurred in measuring the sample with NTA. The nominal size of sample O is 30-50 nm, but the hydrodynamic diameter mean measured by NTA is around 75 nm in pristine water. The NTA mode values, however, trended toward a more accurate representation of the nominal size of the AgNPs, than the mean values, Figures 3 and 4, but still misrepresented the nominal sizes.

**5.5 Environmental Media.** The diameter of the AgNPs in environmental media, just like in pristine waters, can be expected to differ depending on several factors, including the measurement technique, the thickness of the initial capping agent, and in the case of environmental waters, the thickness of the natural organic matter (NOM) layer sorbed onto the AgNPs. The capping agent and NOM density on the surface and ability of NOM molecules to

competitively displace the initial capping agent also impacted the observed size by NTA and DLS.

For example, when in MHRW, particle agglomeration, as seen by the NTA mean diameter, was around 130 nm, when the nominal size of the nanoparticle was reported to be 10 nm (Figure 3b). However, the NTA mode measured more accurately, in most cases, the nominal sizes of AgNPs in MHRW (Figure 3b). With the exceptions to this observation in that the NTA mode size was off significantly in samples M and O (Figure 4b). Again, the errors of the NTA mode measurements were probably due to the formation of agglomerates between AgNPs and the NOM in the water sample.

The results in Table 2 suggest that the types of NOM, as well as, the length of time between dispersion preparation and examination, are critical experimental factors to report alongside size distributions of “stock” AgNPs.

**5.6 Powders vs. Suspensions.** AgNPs received as aqueous suspensions more frequently exhibited a narrow size distribution compared with the powder stock sources, which required a subsequent dispersion step. The quality of dispersions formed from the dry powders varied widely, depending upon many factors, including those associated with the application of ultrasonic energy (e.g., input power, temperature, geometry of the sonicator and solution vessel), the selection or inclusion of capping agents, and solution chemistry (Taurozzi et al. 2011). Recently, protocols addressing how to prepare (Taurozzi et al. 2010), and report the preparation of these powders (Taurozzi et al. 2010) has been published.

There can also be temporal changes to the quality or stability of powder dispersions. For example, serial dilutions of a powder of PVP-capped AgNPs dispersed into DI water were examined by collecting an absorbance spectrum every hour for 96 h after treatment in a bath sonicator and vortexing for approximately one min. This experiment revealed what might typically be reported for an absorbance spectrum over the wavelength range of 300 to 800 nm. At longer wavelengths, there occurred a turnover to a broad absorbance spectrum, which was nearly featureless. This suggested that the sample was very polydisperse, significantly aggregated, in a suspension that settled below the beam-path nearly completely (approximately 90 % loss of absorbance) over a few days.

**5.7 Additional Measurement Methods.** In addition to the information provided above for the methods of nanoparticle detection, other references provided further insights into the use of NTA for screening test purposes.

**5.7.1** Filipe et al., compared NTA with DLS for measurement of nanoparticles and protein aggregates (Filipe et al. 2010). The evaluation was conducted using polystyrene beads (size range of from 60 nm to 1000 nm) in addition to drug delivery and protein aggregate

particles (minimum size of about 1 nm). The evaluation concluded that NTA could "... accurately analyze the size distribution of monodisperse and polydisperse samples. Sample visualization and individual particle tracking are features that enable a thorough size distribution analysis. The presence of small amounts of large (1000 nm) particles generally does not compromise the accuracy of NTA measurements, and a broad range of population ratios can easily be detected and accurately sized."

However, the study also concluded that while the presence of large particles had little impact on sizing accuracy it did reduce the number of small particles detected by the software. The study further noted a lower detection limit for nanoparticles (protein samples) of about 30 nm for NTA and remarked that the analysis can be relatively more time consuming than other nanoparticle measuring techniques, and required optimization steps by a skilled operator.

**5.7.2** Bundschuh et al., describes the Laser-induced Breakdown Detection (LIBD) method for determination of mean particle size and concentration in aqueous samples (Bundschuh et al. 2005). The method was described as capable of detecting particles sizes ranging from about 10 to 1000 nm, and in concentrations in the range from about 1 ng/L up to mg/L. This technique used a high-energy pulsed laser beam to selectively generate plasma on particles and was described as being non-invasive, not requiring sample preparation and allowing on-line measurements. This method did not provide for determination of size distributions and did not allow for differentiation between inorganic, organic and biological particles. A typical on-line measurement can be performed in several minutes.

**5.7.3** Pergantis et al., coupled hydrodynamic chromatography (HDC) with inductively coupled plasma–mass spectrometry (ICP-MS) for detecting metal-containing ENMs in environmental matrices (Pergantis 2010). HDC is suitable for sizing nanoparticles within the range of 5 to 300 nm, and has rapid analysis time. HDC coupled with the selectivity and sensitivity of ICP-MS, makes this a promising analytical tool for investigating the fate of nanoparticles.

However, one serious drawback with this technique was that it could not distinguish between a large concentration of nanoparticles, containing a small metal fraction, and a low concentration of the same size nanoparticles, with a high metal fraction. To address this, Pergantis et al., investigated the use of single particle (SP) ICP-MS coupled with the HDC (Pergantis et al. 2011). SP-ICP-MS demonstrated the capability being able to simultaneously determining the concentration of metal-containing nanoparticles and measuring the metal mass in individual AgNP

## 6.0 CONCLUSIONS

**6.1** Based on the results of the research, and reviews of current literature, no one testing technique appeared to provide all of the desired measurement information for a screening test to characterize nanoparticles in the laboratory and environmental water samples. The evaluations suggest that the most effective approach would be to employ at least two complementary methods, a microscopy-based (e.g., SEM, TEM, AFM), and a dynamic light-based method (e.g., NTA, DLS), to meet desired criteria.

**6.2** The laser detection technique using NTA appeared to be a good candidate for one of the two complementary methods noted above. The method described in this report used the NanoSight Ltd LM-20 instrument system together with the NTA software to perform the analyses. In this evaluation, the major advantages of this system were that it provided data on nanoparticle sizes and distribution, and that it allowed direct/real-time visualization of the nanoparticles in a sample.

Other observations regarding NTA are that it:

1. Does not appear to be materials-specific with regard to ENMs. It has been shown to perform satisfactorily when analyzing metallic nanoparticles.
2. Is size-sensitive above approximately 20 nm and can analyze multi-sized particle dispersions. Results are less reliable below 20 nm to 10 nm with best results only for particles with a high refractive index (e.g., Ag and Au).
3. Has detection capabilities that do not seem to be hampered by surface coatings, with the limitation that indicated particle sizes may be for individual particles or for particle agglomerates.
4. Have detection capabilities that should not be affected by agglomeration (results would be expected to show an increase of larger size particles in the sample distribution).
5. Has been tested for nanoparticles only (ionic form testing has not been addressed).
6. Has screening level capabilities that are satisfactory for two “reconstituted” (contaminated) samples. Additional testing will be needed for a wider array of samples.
7. Have levels of sensitivity to discriminate between ENMs and other materials that will require further evaluation to determine whether nanoparticle materials (besides Ag) can be differentiated from other natural and/or man-made particles in solution.

In the final analysis, the selection of a complementary screening technique to NTA should be made with regard to the additional considerations noted below.

## 7.0 FUTURE RECOMMENDATIONS

For this report, detection of nanoparticles using NTA was the primary focus. There are a substantial number of related items which were beyond the scope of this report which have to do not only with the ability to detect ENMs, but also with the potential toxicity of detected materials in environmentally relevant suspensions.

**7.1 Additional Considerations.** Related physical and chemical features that are important to the full characterization of nanoparticles are as follows:

1. **Nanoparticle Material Composition.** The evaluations described in this report were conducted using silver nanoparticles (AgNPs) because they are widely used in commercial products today. However, there are a number of other nanoparticle materials in various forms in current use (e.g., Au, Ti, Al, C). Therefore, any nanoparticle screening test should address these types of nanomaterials as well. Additional testing of the NTA method using nanoparticles other than Ag (e.g., Au, Ti, Al, and/or C) should be performed. NTA performance for Ag samples has initially been established for “pristine conditions” and for one NOM containing water. The NTA method worked well for particles with a high refractive index (i.e., AgNP) and testing other nanomaterials with different refractive indices should be instructive, particularly with regard to detecting smaller nanos (in the range of 1 to 10 nm).

2. **Other variables.** For toxicity evaluation, nanoparticle sizes, concentrations and exposure times may all be relevant variables.

3. **Particle coatings and capping agents.** Particle coatings combined with capping agents and other surface coatings should be tested with NTA. As was seen in the data tables, capping agents and other surface coatings, such as citrate, starch and polyvinylpyrrolidone can affect test measurement results since some test methods react more strongly to particle core (uncoated) size as opposed to overall size. There are data suggesting that the use of coatings can affect the toxicity of the NPs compared to the pure form (Allen et al. 2010). Several of the nanoparticle types tested have these properties, but further attention is warranted.

4. **Aggregation and agglomeration tendencies.** There are data available that indicates that the AgNPs have a tendency to agglomerate in solution and settle out (Fabrega et al. 2011). This could affect availability, transport mode and eventually the potential toxicity of the AgNPs, and should be addressed.

5. **Ag Nanoparticles vs. Ionic Form.** There are indications that both forms can be toxic (Kennedy et al. 2010) and any tendencies for changes in form due to chemical effects or other factors need to be better understood.

6. **Sample matrix composition.** The samples tested by NTA were limited to two standardized dispersions (i.e., DI water, MHRW). These provided a set of “baseline” conditions, but a wider variety of contaminated waters (both field-obtained and laboratory samples) should be evaluated.

7. **Sensitivity and discrimination capabilities.** The ability of a screening test to distinguish between potentially toxic nanoparticles and other naturally occurring and/or man-made (non-toxic) particles may be a significant challenge. Drinking waters, for example, contain substantial nano-sized particulates in comparable concentrations that may, or may not, be toxic (Kaegi et al. 2008). Also, the possibility that particulates (other than those of specific interest in the nanoparticle screening test) could be toxic should not be ignored.

8. **Additional Testing.** Testing of additional contaminated samples with a wider range of contaminants (both sizes and types) is warranted. Results could be compared, using the same samples, with results from SEM, TEM and/or AFM methods. The microscopy-based techniques are effective for smaller nanoparticle sizes. They appear to be especially responsive to particle core size and not necessarily to overall size, such that sample selection and/or preparation will be important.

## **TABLES**

**Table 1 Mean sizes of AgNPs under pristine conditions**

**Table 2 Mean sizes of AgNPs in MHRW**

**Table 1.** Mean sizes of AgNPs in DI water.

Sample Code	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
Nominal Size (nm)	10	20	20	20	40	60	80	100	10	10	50	50	1-10	10-15	30-50
Capping Agent	citr.	citr.	citr.	citr.	citr.	citr.	citr.	citr.	PVP	PVP	PVP	PVP	unk.	starch	oleic acid
Form Received	susp.	susp.	susp.	susp.	susp.	susp.	susp.	susp.	susp	powd	powd	susp.	susp	susp.	powd.
DLS mean Z-avg (nm)	22.7	35.1	50.0	30.8	46.3	84.1	114	98.7	23.8	110	146	54.4	547	155	103.0
DLS std dev (nm) <sup>[1]</sup>	1.9	0.2	1.6	1.2	0.6	0.9	1.9	1.2	1.0	6.7	1.0	0.8	7.5	1.2	1.3
DLS mean PI	0.377	0.536	0.354	0.095	0.292	0.155	0.113	0.073	0.374	0.416	0.231	0.195	0.597	0.280	0.332
AFM mean height (nm)	10.8	21.3	15.6	23.6	39.5	44.7	56.3	39.5	11.0	12.4	104	46.4	7.9	10.2	97.3
AFM std dev (nm) <sup>[2]</sup>	2.2	3.9	6.5	4.6	18.9	37.5	50.4	40.6	2.2	19.9	91.8	8.5	5.4	5.4	69.9
AFM # NPs counted	107	102	100	100	131	71	44	100	102	100	102	100	102	106	105
<b>NTA Mean Size (nm)<sup>[3]</sup></b>	<b>28.8</b>	<b>44.0</b>	<b>48.2</b>	<b>28</b>	<b>74</b>	<b>106</b>	<b>111</b>	<b>130</b>	<b>54</b>	<b>---</b>	<b>---</b>	<b>39</b>	<b>195</b>	<b>18.2</b>	<b>75</b>
<b>NTA Mode Size (nm)</b>	<b>20.0</b>	<b>33.2</b>	<b>39.0</b>	<b>51</b>	<b>62</b>	<b>87</b>	<b>106</b>	<b>122</b>	<b>26</b>	<b>---</b>	<b>---</b>	<b>30</b>	<b>21.8</b>	<b>16.6</b>	<b>38</b>
<b># completed Tracks</b>	<b>125</b>	<b>520</b>	<b>645</b>	<b>38</b>	<b>428</b>	<b>371</b>	<b>620</b>	<b>175</b>	<b>106</b>	<b>---</b>	<b>---</b>	<b>106</b>	<b>150</b>	<b>125</b>	<b>10.16</b>
TEM Mean size (nm)	8.0	20.3	20.9	29.2	---	---	---	95.6	9.0	---	---	49.8	3.6	13.2	46.2
TEM std dev (nm) <sup>[2]</sup>	2.1	2.2	3.1	3.0	---	---	---	11.8	2.3	---	---	5.2	1.4	6.8	10.4
TEM # NPs counted	81	80	80	51	---	---	---	75	74	---	---	74	80	NA	82
SAXS Mean Size (nm) <sup>[4]</sup>	13.6	21.3	---	27.9	---	---	112	98.4	17.0	---	---	48.7	---	11.1	---
SAXS Peak % of Total Vol. Frac. <sup>[5]</sup>	99	83	---	71.2	---	---	100	100	95	---	---	100	---	82	---
SAXS Total Vol. Frac. of All AgNPs (x10 <sup>-6</sup> )	2.34	1.90	---	6.86	3.87	8.49	4.29	0.25	0.38	---	---	2.23	---	33.2	---
UV-Vis $\lambda_{max}$ (nm)	389	403	401	407	432	489	471	486	394	432	---	424	430	408	423
UV-Vis peak Abs <sup>[6]</sup>	3.42	2.2	NA	0.96	0.54	0.3	0.19	0.75	76.16	NA	---	79.42	17.64	3.86	7.38
Abs/mass Ag ( $\mu\text{g mL}^{-1}$ )	0.17	0.11	NA	0.048	0.027	0.015	0.094	0.037	0.076	NA	---	0.079	0.012	0.24	0.008

Nominal diameters or size ranges are provided by the vendor. [1] DLS  $Z_{avg}$  diameters are the mean of no less than five experiments performed under repeatability conditions, with one standard deviation uncertainty from the mean. [2] AFM heights and TEM diameters are the mean and one standard deviation of all AgNPs sized. [3] NTA mean and mode diameters are based on the number of AgNPs sized, or number of completed tracks. [4] USAXS diameters are the mean size of the most significant size distribution peak, *i.e.*, the peak with the greatest percentage of the total volume fraction of all AgNPs. [5] An estimated uncertainty of  $\pm 10\%$  should be applied to all USAXS values. [6] UV-vis peak absorbance is calculated for the undiluted stock solution, using the dilution factor for solutions that provided measured absorbance values between 0.05 and 2.0. UV-Vis lambda max is the wavelength in nm of peak absorbance, and the absorbance per mass of AgNP is in units of  $\mu\text{g mL}^{-1}$ . Dashes indicate no measurements using that technique were made on that sample.

**Table 2.** Mean sizes of AgNPs in MHRW.

Sample Code	A	B	D	E	F	G	H	I	J	K	L	M	O
Nominal Size (nm)	10	20	20	40	60	80	100	10	10	50	50	1-10	30-50
DLS mean Z-avg (nm)	33	49.9	39.0	51.6	90.8	121	196	146	228	111	43.8	127	251
DLS std dev (nm)	7.3	0.7	0.5	0.5	0.7	3.2	17.4	133	38.1	3.5	0.6	3.0	233
AFM mean height (nm)	13.3	21.3	24.3	33.0	29.1	35.7	111	11.4	32.7	37.5	38.0	4.8	149
AFM std dev (nm)	4.6	5.3	4.6	23.0	24.8	44.1	22.6	4.0	38.8	49.8	14.1	3.8	87.6
AFM # NPs counted	101	100	102	37	26	18	4	100	56	8	55	102	82
<b>NTA Mean Size (nm)</b>	<b>133</b>	<b>42</b>	<b>56</b>	<b>60</b>	<b>85</b>	<b>104</b>	<b>104</b>	---	---	---	<b>52</b>	<b>62.7</b>	<b>131</b>
<b>NTA Mode Size (nm)</b>	<b>20</b>	<b>52</b>	<b>53</b>	<b>56</b>	<b>81</b>	<b>100</b>	<b>97</b>	---	---	---	<b>46</b>	<b>52.7</b>	<b>114</b>
<b># completed Tracks</b>	<b>40</b>	<b>1128</b>	<b>1829</b>	<b>1653</b>	<b>7</b>	<b>403</b>	<b>1134</b>	---	---	---	<b>1869</b>	<b>646</b>	<b>293</b>

Sample codes and uncertainties follow the same conventions described in Table 1.

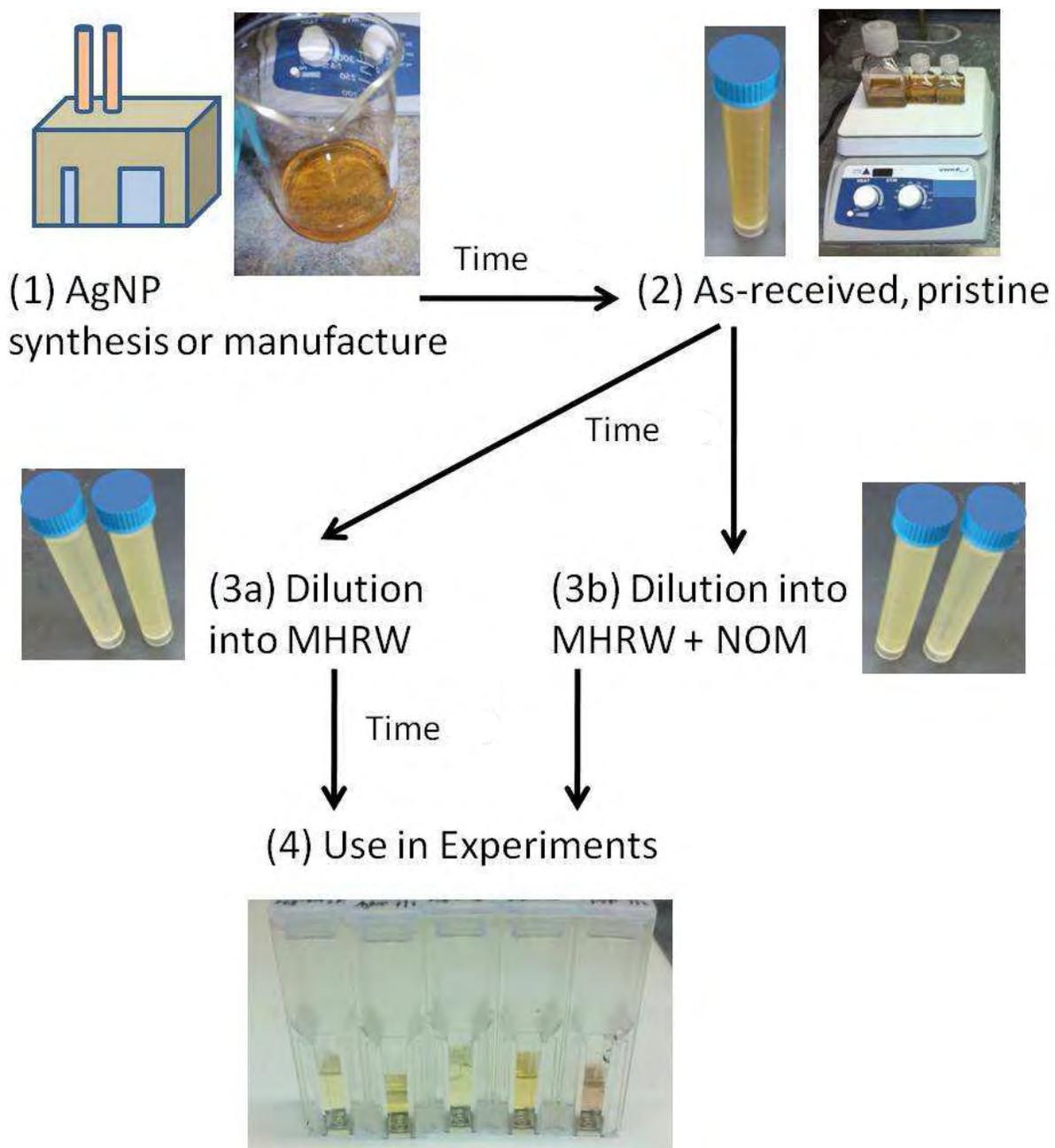
## **FIGURES**

**Figure 1** Illustration of common measurement points of “stock” AgNPs

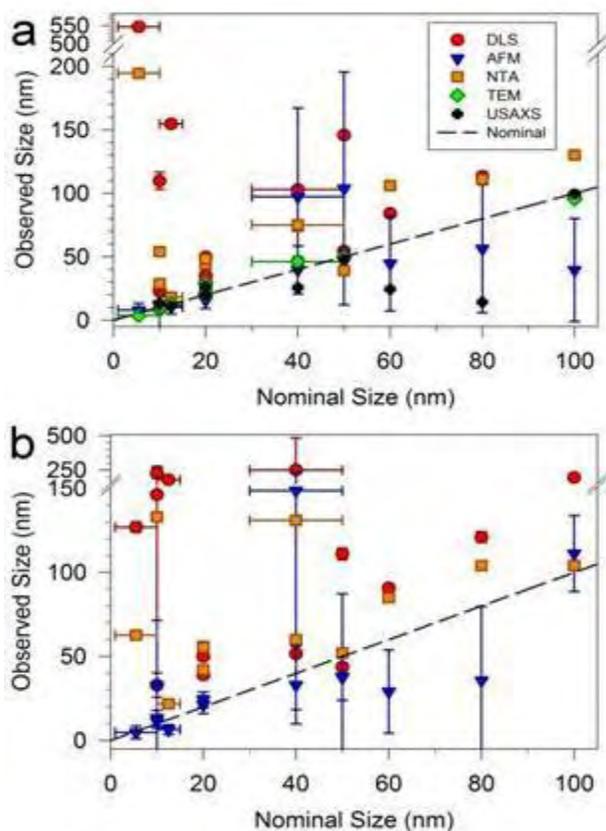
**Figure 2** Measured particle mean size vs. “nominal” size reported by manufacturer

**Figure 3** Distribution of NTA mean and mode measurements in (a) pristine and (b) MHRW Waters – Samples A to H

**Figure 4** Distribution of NTA mean and mode measurements in (a) pristine and (b) MHRW Waters – Samples L - O

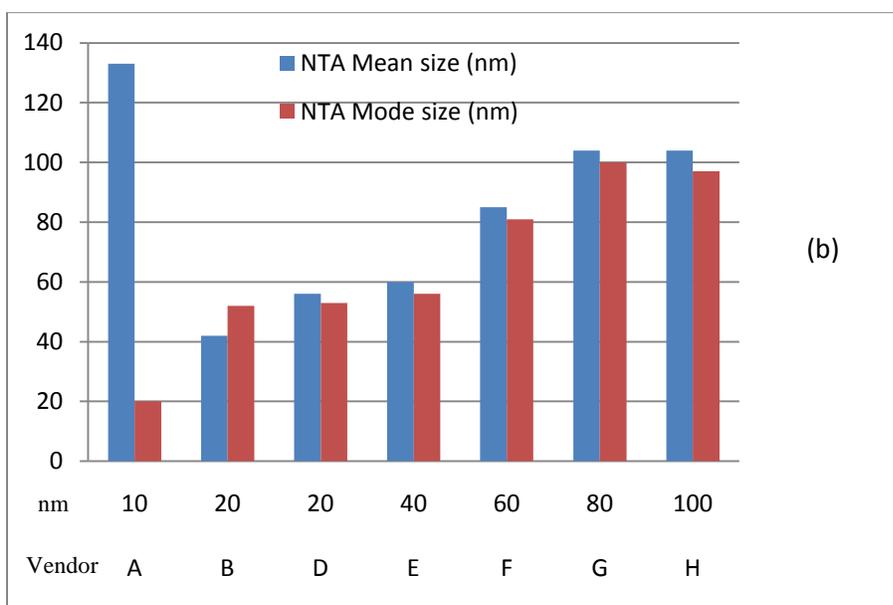
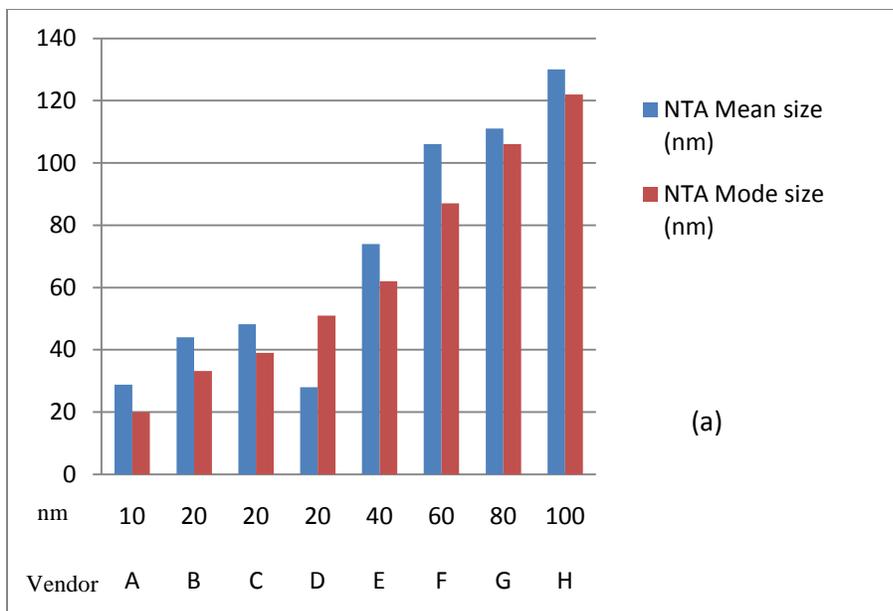


**Figure 1 Illustration of common measurement points of “stock” AgNPs** - Scheme illustrating some of the common points that measurements of “stock” AgNPs could take place. The variables of time between points and storage conditions can affect the stability and degradation kinetics of the AgNPs and thus the observed size and size distribution of the same lot of material.

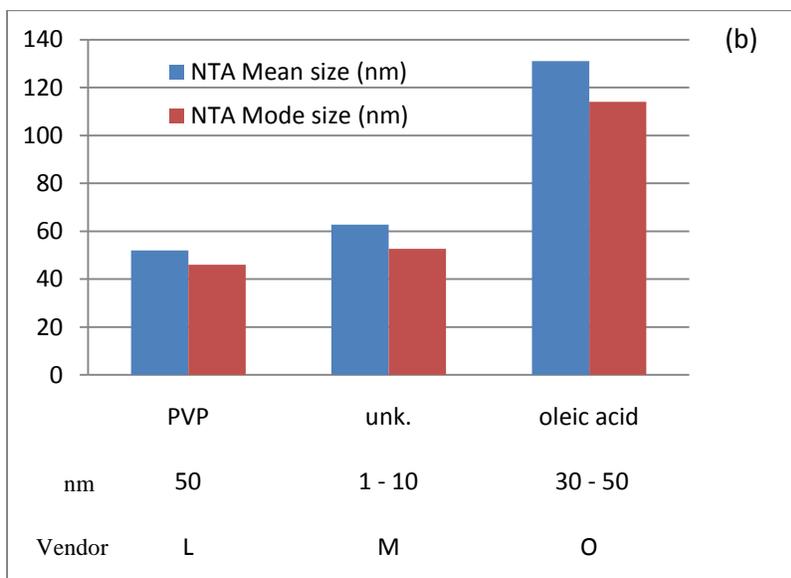
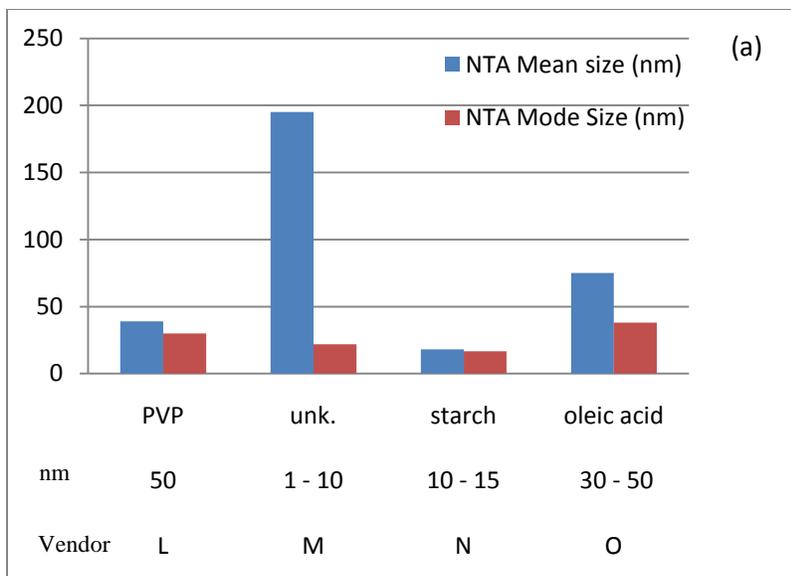


**Figure 2 Measured particle mean size vs. “nominal” size reported by manufacturer.**

Observed mean size by measurement technique plotted against the “nominal” size reported by manufacturer for (a) pristine conditions and (b) dilution into MHRW. Samples A-H are plotted in (a) and (b). All panels use same legend of points and lines shown in (a). Vertical bars in observed size (y-axis) follow conventions described in the experimental section, keeping in mind that error bars for different techniques have different meanings, from precision of the measurement to width of the distribution; refer to Tables 1-2 for values smaller than symbols. Horizontal bars in nominal size (x-axis) represent the range provided by the source, where appropriate.



**Figure 3.** NTA Mean and Mode sizes of citrate-capped AgNPs in (a) pristine water; and (b) MHRW.



**Figure 4.** NTA mean and mode sizes of different capped AgNps in (a) pristine water and (b) MHRW.

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