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LC-MS/MS Measurement of Nanomaterial-Induced DNA Modifications in Isolated DNA

Version 1.0

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FOREWORD

This special publication is one in a series stemming from the National Nanotechnology Initiative (NNI) Nano-EHS Research Strategy which identified Nanomaterial Measurement Infrastructure as one of the essential areas of research needed in order to develop an effective risk assessment and management plan regarding various aspects of nanotechnology in consumer products as it pertains to human health, exposure and the environment. The National Institute of Standards and Technology (NIST) was identified as a lead agency in the development of measurement strategies for the robust development to assess the potential effects of engineered nanomaterials and their fate in the environment. One important endpoint for measuring the potential human health and ecological effects of nanomaterials is the extent of modification that may occur on DNA bases, nucleosides or nucleotides. These modifications are considered to be DNA damage and may be relevant for the risk assessment of nanomaterials in biological systems.

The current protocol presents a method to measure DNA 2'-deoxynucleoside lesion levels using isotope-dilution liquid chromatography/tandem mass spectrometry. Updates to this protocol may be released in the future. Visit http://nist.gov/mml/np-measurement-protocols.cfm to check for revisions of this protocol, or new protocols in the series. We also encourage users to report citations to published work in which this protocol has been applied.

1. Introduction

Making accurate measurements of the environmental fate and environmental and biological effects of engineered nanomaterials (ENMs) is critical for reliable risk assessment of these materials. It has been shown that ENMs can induce DNA damage through oxidative stress in living systems and/or through direct binding of ENMs to DNA. However, the unique behaviors of ENMs may cause measurement artifacts during the determination of their DNA damaging potential [1]. For example, results from several DNA damage studies using the traditional Comet assay have reported artifacts from the presence of ENMs [2-6]. Potential explanations for this phenomenon are that ENMs may induce DNA damage during processing after the exposure period has concluded or that the ENMs may be in the Comet tail and mistaken for DNA. These types of measurement artifacts preclude the accurate measurement of DNA damage and prevents researchers from clarifying the fundamental mechanisms of ENM induced DNA modifications [2].

In this document, we describe a protocol to quantitatively measure a range of modified 2'-deoxynucleosides in DNA using liquid chromatography/tandem mass spectrometry (LC-MS/MS) with isotope-dilution and NIST standard reference material (SRM) 2396 (Oxidative DNA Damage Mass Spectrometry Standards) for the measurement of 8-hydroxy-2′-deoxyguanosine (8-OH-dGuo); the other four analytes that can be measured (described below) must utilize stable isotope-labeled internal standards that are synthesized separately. This method circumvents many of the artifacts observed in the Comet DNA strand break assay by directly quantifying molecular level DNA damage (i.e., DNA lesion) for a range of oxidatively induced damage products. Isotope-dilution mass spectrometry methods have been recently utilized by our laboratory to successfully measure DNA damage both *in vitro* and *in vivo* caused by gold nanoparticles [7], copper oxide nanoparticles [8], single-wall carbon nanotubes [9], iron oxide nanoparticles [10], titanium dioxide nanoparticles [11] and silver nanoparticles [12].

2. Principles and Scope

This protocol is proposed for the measurement of DNA lesion levels using LC-MS/MS with isotope-dilution [13] after exposure of isolated DNA to ENMs. Methods for determination of DNA lesion levels after cellular or whole organism exposure to ENMs are similar but are not described in the present protocol. Not all possible forms of DNA damage can be measured by this approach. This protocol focuses specifically on oxidatively-induced damage to DNA 2'-deoxynucleosides. The methodology, and specifically the instrumentation described in the present protocol, is based on the accurate measurement of DNA base lesions using a triple quadrupole mass analyzer in multiple reaction monitoring (MRM) mode (see Table A). The MRM transitions for each modified 2'-deoxynucleoside are specifically given in section 6.2 below. Usage of the isotopically labeled internal standard, 8-hydroxy-2′-deoxyguanosine-$^{15}N_5$ (8-OH-dGuo-$^{15}N_5$) from SRM 2396 enables quantification of 8-OH-dGuo. Other DNA lesions such as (5′*S*)-8, 5′-cyclo-2′-deoxyguanosine (*S*-cdGuo), (5′*R*)-8, 5′-cyclo-2′-deoxyguanosine (*R*-cdGuo), (5′*S*)-8, 5′-cyclo-2′-deoxyadenosine (*S*-cdAdo) and (5′*R*)-8, 5′-cyclo-2′-deoxyadenosine (*R*-cdAdo) can also be measured if the isotope-labeled analogues of these lesions [(5′*S*)-8, 5′-cyclo-2′-deoxyguanosine-$^{15}N_5$, (5′*R*)-8, 5′-cyclo-2′-deoxyguanosine-$^{15}N_5$, (5′*S*)-8, 5′-cyclo-2′-deoxyadenosine-$^{15}N_5$ and (5′*R*)-8, 5′-cyclo-2′-deoxyadenosine-$^{15}N_5$, are synthesized from commercially available dGTP-$^{15}N_5$ and dATP-$^{15}N_5$, respectively [14, 15].

Table A DNA lesions detected and quantified by different MS procedures.

Lesion #	GC/MS (bases by SIM)	GC-MS/MS (bases by MRM)	LC-MS/MS[1] (nucleosides by MRM)
1	FapyAde	FapyAde	8-OH-dGuo
2	FapyGua	FapyGua	*R*-cdAdo
3	8-OH-Ade	8-OH-Ade	*S*-cdAdo
4	5-OH-Cyt	5-OH-Cyt	*R*-cdGuo
5	5-OH-Ura	5-OH-Ura	*S*-cdGuo
6	5-(OHMe)Ura	5-(OHMe)Ura	
7	ThyGly	ThyGly	
8	5-OH-5MeHyd	5-OH-5MeHyd	
9	8-OH-Gua	8-OH-Gua	

1. Nucleoside lesions detected and quantified in the present protocol.

3. Terminology

This protocol complies with definitions relevant to nanotechnology as set forth in the ASTM International standard E2456 [16] and is consistent with the draft standard ISO TS 80004-1:2010 [17].

nanoparticle—sub-classification of ultrafine particle that is characterized by dimensions in the nanoscale (i.e., between approximately 1 nm and 100 nm) in at least two dimensions; also referred to as "nano-object" in ISO TS 80004-1:2010 [17].

primary particle — the smallest discrete identifiable entity associated with a particle system; in this context, larger particle structures (e.g., aggregates and agglomerates) may be composed of primary particles.

aggregate — a discrete assemblage of primary particles strongly bonded together (i.e., fused, sintered, or metallically bonded).

> *Note—The adjective "primary", when used in conjunction with the term aggregate, is employed in the present context to indicate the smallest achievable dispersed particle entity.*

agglomerate—assemblage of particles (including primary particles and/or smaller aggregates) held together by relatively weak forces (e.g., van der Waals, capillary, or electrostatic), that may break apart into smaller particles upon further processing.

> *Note—Although we define them as distinct entities, the terms aggregate and agglomerate have often been used interchangeably to denote particle assemblies.*

dispersion—used in the present context to denote a liquid (aqueous) in which particles are homogeneously suspended, or the process of creating a suspension in which discrete particles are homogeneously distributed throughout a continuous fluid phase; implies the intention to break down agglomerates into their principal components (i.e., primary particles and/or aggregates).

4. Materials and equipment

4.1 Reagents

4.1.1 NIST SRM 2396

4.1.2 Distilled and deionized ≅18 MΩ water (ddH_2O), e.g., Millipore Milli-Q; sterile DNase/RNase-free water is recommended (e.g., Ambion, non-DEPC treated)
4.1.3 Dried genomic DNA (e.g., calf thymus DNA, Sigma-Aldrich) or oligomeric DNA (e.g., Integrated DNA Technologies)
4.1.4 Absolute anhydrous ethanol, (e.g., Sigma-Aldrich, <0.005 % water)
4.1.5 Acetonitrile (e.g., Sigma-Aldrich with a minimum purity of 99.9 %, anhydrous)
4.1.6 10 mmol/L Tris-HCl in ddH_2O adjusted to pH 7.5
4.1.7 1 mole/L sodium acetate containing 45 mmol/L zinc chloride in ddH_2O adjusted to pH 6.0
4.1.8 Nuclease P1 (e.g., U.S. Biological)
4.1.9 Phosphodiesterase I (e.g., Sigma-Aldrich)
4.1.10 Alkaline phosphatase (e.g., Roche Diagnostic)
4.1.11 ENMs (e.g., NIST SRM 8011, 8012, 8013, etc.)

4.2 Materials

4.2.1 15 mL centrifuge tubes
4.2.2 Glassware for making ICP-MS measurements
4.2.3 Calibrated pipettes and disposable tips
4.2.4 Quartz cuvettes for UV/Vis measurements
4.2.5 Drierite Desiccant
4.2.6 2 mL glass autosampler vials with caps and low volume (300 µL) inserts
4.2.7 3000 Da molecular weight cut-off centrifugal filters
4.2.8 Chelex 100 resin (e.g., Sigma-Aldrich)

4.3 Equipment

4.3.1 Fixed angle refrigerated centrifuge that is capable of spinning up to 20 000 x g and which is suitable for 2 mL Eppendorf sample tubes
4.3.2 Double beam UV/Vis absorbance spectrophotometer or another instrument capable of quantifying DNA concentrations
4.3.3 Speed Vac system capable of holding 2 mL Eppendorf sample tubes or 2 mL glass autosampler vials or a vacuum desiccator
4.3.4 Horizontal shaker with speed control
4.3.5 -20 ºC freezer
4.3.6 3500 MWCO dialysis membrane
4.3.7 2 mL Eppendorf sample tubes
4.3.8 Triple quadrupole liquid chromatography/mass spectrometry (LC-MS/MS) system with an electrospray ionization source. The system should have a mass range of 50 Da to 1500 Da, the capacity to select high resolution precursor ions of less than or equal to 0.2 Da, the ability to switch ion polarity in less than 0.5 s and the capacity to perform accurate mass measurements on known and unknown analytes with mass assignment stabilities within ± 0.025 Da over a 24 h period
4.3.9 Single quadrupole inductively coupled plasma/mass spectrometry (ICP/MS) system with nickel cones and a Conikal nebulizer or a suitable single quadrupole inductively coupled plasma/optical emission spectrometry (ICP/OES) system

5. Treatment of and removal of ENMs from laboratory prepared DNA stock solutions.

5.1 Preparation of DNA stock solutions

5.1.1 To a known mass of dried DNA in a 15 mL plastic centrifuge tube, add a known volume fraction of ddH_2O so that the final concentration of DNA in the tube is ≤ 500 µg/mL.

5.1.2 Place the tube on a orbital rotator in a 4 °C refrigerator and gently mix the DNA solution for 72 h to ensure complete solubilization.

5.1.3 Treat the solubilized DNA with Chelex 100 resin to remove heavy metals and divalent transition metals.

5.1.4 Dialyze (3500 MWCO membrane, 25 mm to 30 mm diameter) the DNA against ddH_2O at 4 °C for 24 h and change the water at least twice during this time period.

5.1.5 Determine the concentration of the solubilized DNA using UV/Vis spectrophotometry (1 absorbance unit equals 50 µg/mL double-stranded DNA at 260 nm). For the stock DNA solution, it is recommended that the user also check the purity of the DNA solution by quantitatively evaluating the total RNA and protein levels in the sample. An estimate of RNA contamination can be quickly obtained by evaluating the 260 nm/280 nm ratio for the sample. A ratio of 1.8 to 2.0 indicates pure DNA while values higher than 2.0 suggests RNA contamination. Total RNA contamination can be quantitatively evaluated using the Qubit fluorescence assay [18]. Proteins absorb at 280 nm but the absorbance at 280 nm has a minimal effect on the 260 nm/280 nm ratio due to the much higher extinction coefficient of nucleic acids at both 260 nm and 280 nm. However, protein levels in the DNA solution can be quantitatively assessed using either the Lowry [19] or Bradford [20] protein assays.

5.1.6 Store the DNA stock solution at 4 °C until needed or proceed directly to treating the DNA with ENMs based on the experimental design.

5.2 Treatment of DNA solutions with ENMs

5.2.1 To 2 mL Eppendorf sample tubes, add an appropriate volume of DNA stock solution so that the total mass of DNA in each tube is 100 µg.

5.2.2 Add a specified volume of dispersed ENM and additional dispersant into each sample tube so that an appropriate range of ENM sample concentrations are obtained.

5.2.3 Prepare both positive and negative control samples using an identical procedure, as well as a dispersant-only control sample. All test and control samples should be prepared in triplicate. Prepare extra sets of triplicate control samples that are both unspiked and spiked (50 mg/L) with the highest ENM concentration under investigation. These samples will be used for verification/quantification of ENM removal via ICP-MS or ICP-OES (see below).

5.2.4 Treat all of the samples as necessary (i.e., expose to light/dark, heat/cold, adjust pH, sonicate, etc.,) for the specified period of time based on the experimental design.

5.3 Removal of ENMs from DNA solutions

5.3.1 Removal of the ENMs from the DNA samples is performed based upon centrifugation followed by ICP-MS quantitative verification of ENM removal. In some instances, it may be appropriate to utilize ICP-OES analyses if elemental detection sensitivity is not a limiting factor.

5.3.2 Centrifuge the samples at 16 000 g (14 000 rpm) for 1 h @ 4 °C to pellet the ENMs. This procedure has been shown to be suitable for metal (i.e., AuNPs [7]) and metal-oxide (i.e., TiO_2 NPs [11]) ENMs with a minimum diameter of 10 nm. Using this centrifugation procedure, ≅ 98% of the ENMs can be removed from solubilized DNA samples. If less than 98 % of the ENMs are removed from the DNA, then one simply increase the centrifugation time. (Appendix shows validation data for removal of AuNPs (Figure 1) and removal of TiO_2 NPs (Table 1). Please see references above for additional details.

5.3.3 Transfer the DNA supernatants into 30 kDa molecular-weight-cutoff (MWCO) centrifugal filter units and centrifuge the samples at 7000 g for 15 min @ 4 °C.

5.3.4 Wash the filter membrane with ddH_2O then reverse-elute the DNA into a clean 1.5 mL Eppendorf tube.

5.3.5 Determine the concentration of the eluted DNA solution using UV spectrophotometry (1 absorbance unit = 50 µg DNA / mL at 260 nm) and store the collected samples at 4 °C or proceed directly to the addition of the isotopically labeled internal standards to each of the samples (see below). The accuracy of the DNA quantification method can be tested using NIST SRM 2372 (DNA Quantitation Standard).

5.3.6 Verify the level of ENM removal using only the spiked control and unspiked control samples via ICP-MS analysis as follows: add a suitable internal standard to each sample and digest each sample using for example: a 3:1 volume fraction mixture of concentrated HCl/HNO_3 (30 min @ 75 °C with orbital shaking at 60 rpm). This digestion solution will work for AuNPs, but the analyst may need to modify this solution for other metal or metal-oxide-based ENMs.

5.3.7 Cool the control samples down to room temperature and dilute them with 3 % HCl + 1 % HNO_3 as required for analysis. Note: the sample digestion and dilution solutions will change depending on the element of interest.

5.3.8 Perform elemental analysis on the digested control samples using ICP-MS. Based on the elemental calibration response factor, determine the elemental response in the spiked and unspiked control samples. Determine the mean level of ENM removal from the DNA solutions.

6 DNA lesion measurements using LC-MS/MS

6.1 DNA lesion quantification procedure

6.1.1 Transfer the required volume of eluted DNA from the centrifugal filter unit (see above) into a 1.5 mL Eppendorf tube so that the tube contains at least 50 µg of DNA. If there is not enough DNA sample volume to add 50 µg to each sample, add at least 30 µg. It is important to use the same DNA mass for all samples in a sample set.

6.1.2 Add the relevant isotopically labeled internal standards to each vial. We recommend also testing a reference DNA such as calf thymus DNA and an internal standard mixture sample during each sample set analysis to test assay performance. These control samples

are used to confirm the retention time and analyte peak stability of the LC column and to help assess the performance of the mass analyzer. The concentration of internal standard to add for each of the lesions depends on the type of DNA (i.e., isolated DNA, cellular DNA, etc.) and on the dynamic range of the LC-MS/MS instrument. This needs to be tested during preliminary experiments with reference DNA. If too low or too high of an internal standard concentration is used, it may be challenging to integrate the peaks for the internal standard or the calibrated measurement range of the instrument may be exceeded.

6.1.3 Dry the samples using either a Speed Vac or vacuum desiccator. After the samples are dry, either begin the next step or store the samples at 4 ºC in a jar with desiccant.

6.1.4 Add 50 µL of the following freshly prepared buffer to each DNA sample: prepare the buffer with a ratio of 100 µl of the Tris-HCl solution (see 4.1.5) and 2.5 µl of the sodium acetate and zinc chloride solution (see 4.1.6) and scale to the appropriate number of samples. For example, to make enough buffer for 10 samples, add 500 µL of the Tris-HCl solution and 12.5 µL of the sodium acetate and zinc chloride solution.

6.1.5 Add the following enzymes to each sample: 1 µL of nuclease P1 (1 U), 2 µL of phosphodiesterase I (0.001 U), and 1 µL of alkaline phosphatase (16 to 23 U).

6.1.6 Mix the samples by gentle tapping and incubate all samples at 37 °C for 18 to 24 h (heating block or water bath). Do not exceed 24 h.

6.1.7 Transfer each sample to a 3000 molecular weight cut-off centrifuge filter. After adding 50 µL of the sample to the vial, add an additional 200 µL of ddH_2O so that the total volume is 250 µL.

6.1.8 Centrifuge all samples at 15 800 g for ≅ 75 min at 20 ºC and collect filtrate.

6.1.9 Transfer filtrate into a glass sample vial containing a low volume insert and add the cap vial. Make sure that the vial caps are on tightly to prevent oxygen from getting into the sample vials. Try to twist the caps to make sure they are secure.

6.1.10 Analyze the samples using LC-MS/MS methodology [13, 21]

6.2 LC-MS/MS sample analyses

6.2.1 The LC-MS/MS sample analyses are performed on a high resolution triple quadrupole LC-MS/MS system operated in positive electrospray ionization mode.

6.2.2 The LC column is a Zorbax SB-Aq narrow-bore LC column (2.1 mm x 150 mm, 3.5 µm particle size) with an attached Agilent Eclipse XDB-C8 guard column (2.1 mm x 12.5 mm, 5 µm particle size). The column temperature is thermostated at 40 ºC. The gradient LC elution conditions are as follows (all solvent percentages are volume fractions): Mobile phase A = 2 % acetonitrile in water; mobile phase B = 100 % acetonitrile; time program = 0.0 min, 98 % A/2 % B; 18.0 min, 80 % A/20 % B; 18.1 min, 40 % A/60 % B; 20.0 min, 40 % A/60 % B; 20.1 min, 98 % A/2 % B; 25.0 min, 98 % A/2 % B; flow rate = 500 µL/min. MS data is diverted to waste for the first 2 min of the run. The DNA lesions and their stable isotope-labeled analogues are detected in MRM mode after appropriately tuning and optimizing the MS/MS instrument and analyte detection parameters. Each manufacturer's instrument will have a different detection sensitivity for the analytes and thus the analytes will have to be tuned independently. Typical instrument parameters that need to be tuned and optimized for the analytes are the following: analyte dwell time, spray voltage, tube lens offsets, analyte collision energies,

vaporizer temperature, capillary temperature, sheath gas pressure, auxiliary gas pressure, collision gas pressure, scan width, scan dwell time and MS/MS run time. The relevant MRM mass transitions are *m/z* 284 → *m/z* 168 and *m/z* 289 → *m/z* 173 for unlabeled and labeled 8-OH-dGuo, respectively; *m/z* 250 → *m/z* 164 and *m/z* 255 → *m/z* 169 for unlabeled and labeled *R*-cdAdo and *S*-cdAdo, respectively and *m/z* 266 → *m/z* 180 and *m/z* 271 → *m/z* 185 for unlabeled and labeled *R*-cdGuo and *S*-cdGuo, respectively.

6.2.3 The MRM current profiles are integrated using appropriate peak integration software and the measured lesion levels are determined using the MRM area ratios from the DNA lesion of interest and its labeled analogue in conjunction with the known amount of the labeled analogue initially spiked into each sample.

7 Abbreviations

ENM	engineered nanomaterial
8-OH-dGuo	8-hydroxy-2□-deoxyguanosine
R-cdAdo	(5´*R*)-8, 5´-cyclo-2´-deoxyadenosine
S-cdAdo	(5´*S*)-8, 5´-cyclo-2´-deoxyadenosine
R-cdGuo	(5´*R*)-8, 5´-cyclo-2´-deoxyguanosine
S-cdGuo	(5´*S*)-8, 5´-cyclo-2´-deoxyguanosine
LC-MS/MS	liquid chromatography/tandem mass spectrometry
ENM	engineered nanomaterial
NP	nanoparticle
MRM	multiple-reaction-monitoring
SRM	standard reference material
UV/Vis	ultraviolet-visible

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Appendix

1. Removal of Engineered Nanomaterials from DNA Solutions Using Centrifugation Techniques

In the present study, we are strictly concerned with the separation of one discrete size and shape of ENM from a solution matrix of completely solubilized ct-DNA in aqueous buffer. Based on the constant buoyancy and density of the solution matrix, we can utilize the centrifugal force from a fixed-angle rotor centrifuge to separate the ENMs from the DNA solution. We are able to effect an almost 100 % separation of ENMs from the DNA solution due to the fact that the DNA solution has a lower density (ρ) than the density of the relevant ENMs (ρ_{DNA} = 1.7 g/cm^3 and ρ_{AuNPs} = 19.3 g/cm^3, $\rho_{TiO2\ NPs}$ = 4.3 g/cm^3) [22-25]. Therefore, the ENMs can be forced to sediment at the bottom of the DNA solution using appropriate centrifugation speeds and times. When the centrifugation speed is held constant, and assuming spherical particles for simplicity, the appropriate centrifugation time for separating the ENM from the DNA solution can be determined empirically or it can be estimated theoretically using the following equations [26]:

(1) $$v_t = \frac{\omega^2 r(\rho_\rho - \rho_i) d_\rho^2}{18\eta}$$

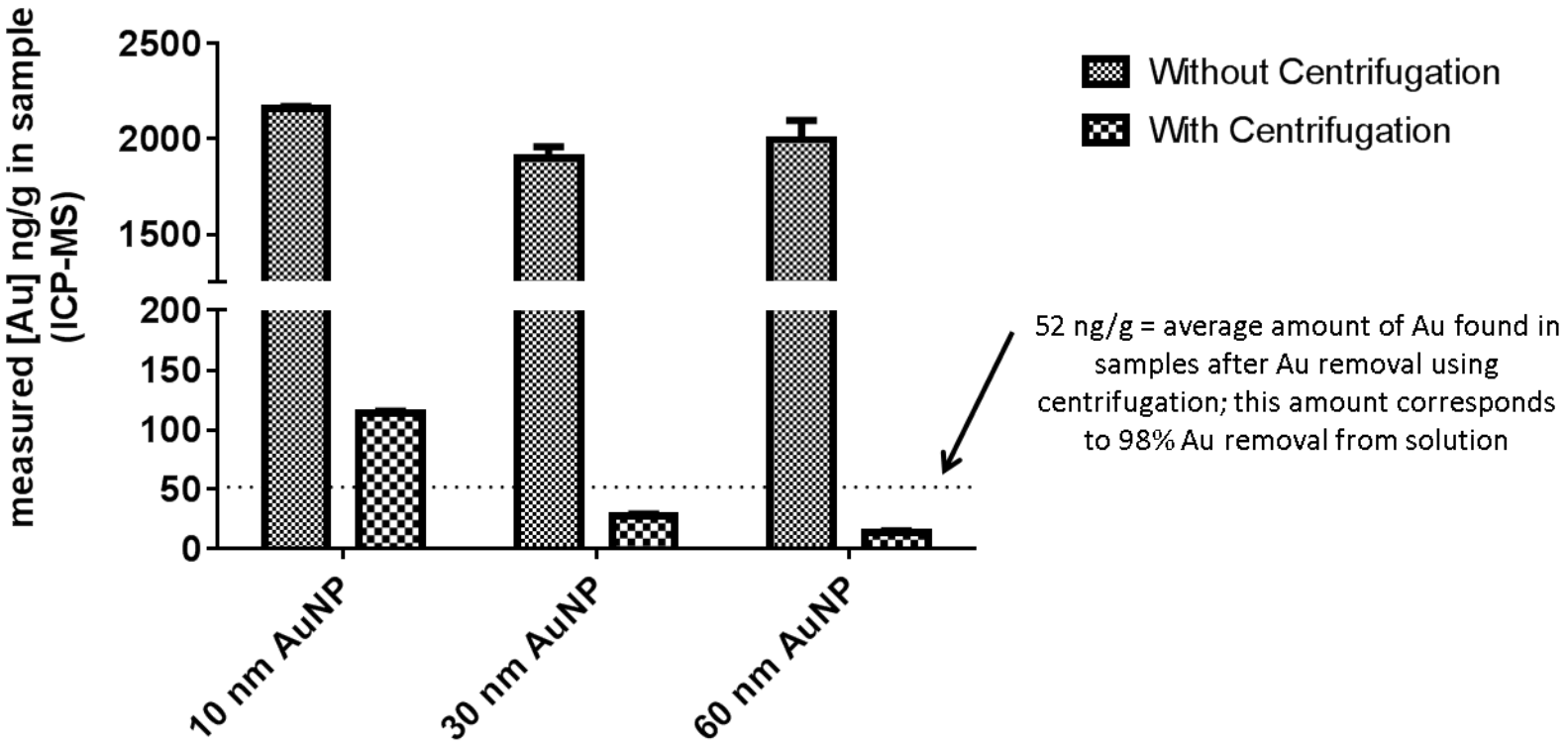
(2) $$v_t = \frac{d_r}{d_t}$$

(3) $$t_t = \frac{\left[\ln\frac{r_{max}}{r_{min}}\right]}{v_t},$$

where v_t is the terminal velocity, ω^2 is the angular velocity of the rotor, ρ_ρ and ρ_i are the density of the ENM and solution matrix (solubilized ct-DNA), respectively, d_ρ is the diameter of the ENM, η is the viscosity of the solution matrix, r_{max} and r_{min} are the maximum and minimum rotor radii, respectively, and t_t is the total time for complete separation of the ENM from the solution matrix.

2. Figure 1

Results from the removal of 10 nm, 30 nm and 60 nm NIST SRM AuNPs from aqueous buffered solutions containing 500 µg/mL ct-DNA using centrifugation.



3. Table 1

Removal of NIST P25 TiO_2 NPs from 500 µg/mL ct-DNA aqueous buffer solutions using centrifugation.

Sample #	**Detected Level of TiO_2 (µg) after centrifugation**	**Standard Deviation (µg)**	**Expected Level of TiO_2 (µg) before centrifugation**	**%TiO_2 Removed**
1	7.516	0.019	198	96.2
2	3.165	0.028	198	98.4
3	2.109	0.010	198	98.9

Ti was detected in the aqueous ct-DNA samples using ICP-OES and converted to TiO2 levels in the samples. The data shown are for three independently prepared samples that were analyzed 5 times each. The mean percentage of TiO_2 removed was 97.8 % ± 1.4 %.