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

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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 nanomaterial induced DNA damage levels using gas chromatography/mass spectrometry with isotope-dilution. 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 DNA lesions using gas chromatography/mass spectrometry (GC/MS) with isotope-dilution and NIST standard reference material (SRM) 2396 (Oxidative DNA Damage Mass Spectrometry Standards). 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 GC/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 bases. The methodology, and specifically the instrumentation described in the present protocol, is based on the accurate measurement of DNA base lesions using a single quadrupole mass analyzer in selected ion monitoring (SIM) mode (see Table A). The monitored SIM ions for each base lesion are specifically given in section 6.2 below. A similar protocol for the quantification of DNA base lesions that describes the use of a triple quadrupole mass analyzer and multiple reaction monitoring transitions for the measurement of ENM induced DNA damage also exists, but this protocol exists as a separate document. Single quadrupole mass analyzers cost approximately one-third the price of triple quadrupole mass analyzers, therefore, the current methodology is inherently more cost accessible for more users. In addition, the instrumental expertise needed to properly operate and optimize a single quadrupole instrument is not as extensive as the expertise required to properly operate a triple quadrupole instrument. Usage of stable isotope-labeled internal standards (ISTDs) from SRM 2396 enables absolute identification and quantification of the following DNA lesions: 4,6-diamino-5-formamidopyrimidine (FapyAde), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), 8-hydroxyadenine (8-OH-Ade), 5-hydroxycytosine (5-OH-Cyt), 5-hydroxyuracil (5-OH-Ura), 5-(hydroxymethyl)uracil (5-(OHMe)Ura), thymine glycol (ThyGly) and 5-hydroxy-5-

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methylhydantoin (5-OH-5-MeHyd). Other important DNA lesions such as 8-hydroxyguanine (8-OH-Gua) can also be measured with this approach by hydrolyzing 8-hydroxy-2-deoxyguanosine-$^{15}N_5$ ISTD in SRM 2396 to 8-hydroxyguanine-$^{15}N_5$ (8-OH-guanine-$^{15}N_5$) or stable isotope-labeled versions of 8-OH-Gua may be synthesized directly [14]. In this procedure, 8-OH-guanine-$^{15}N_5$ is obtained by hydrolysis of 8-hydroxy-2-deoxyguanosine-$^{15}N_5$ with 60% formic acid at 140 °C for 30 min followed by lyophilization. Subsequently, 8-OH-guanine-$^{15}N_5$ is dissolved in 10 mM NaOH and its concentration in solution is determined using UV spectrophotometry prior to use.

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

Lesion #	GC/MS[1] (bases by SIM)	GC-MS/MS (bases by MRM)	LC-MS/MS (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. Base 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 E2456 [15] and is consistent with the draft standard ISO TS 80004-1:2010 *[16]*.
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 [16].
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 $\cong$18 MΩ water (ddH$_2$O), e.g., Millipore Milli-Q; sterile DNase/RNase-free water is recommended (e.g., Ambion, non-DEPC treated)
4.1.3   Dried genomic DNA (e.g., calf thymus DNA, Sigma-Aldrich) or oligomeric DNA (e.g., Integrated DNA Technologies)
4.1.4   Absolute anhydrous ethanol, (e.g., Sigma-Aldrich, <0.005 % water)
4.1.5   Acetonitrile (e.g., Sigma-Aldrich with a minimum purity of 99.9 %, anhydrous)
4.1.6   Fpg/Nth buffer (50 mmol/L sodium phosphate, 100 mmol/L potassium chloride, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.1 mmol/L dithiothreitol (DTT), and adjusted to pH 7.4 using ddH$_2$O)
4.1.7   DNA repair enzymes (e.g., Fpg or Nth, Trevigen)
4.1.8   Liquid nitrogen
4.1.9   Pyridine (e.g., Sigma-Aldrich, stored in a amber glass vial with a few pellets of KOH to help remove water)
4.1.10 Bis(trimethylsilyl)trifluoroacetamide + 1 % trimethylchlorosilane (BSTFA + TMCS, e.g., ThermoScientific Corp)
4.1.11 High purity (99.999 %) nitrogen gas (e.g., Airgas Inc.)
4.1.12 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   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   Vortex mixer
4.3.3   Double beam UV/Vis absorbance spectrophotometer or another instrument capable of quantifying DNA concentrations
4.3.4   Speed Vac system capable of holding 2 mL Eppendorf sample tubes or 2 mL glass autosampler vials or a vacuum desiccator
4.3.5   Horizontal shaker with speed control
4.3.6   Orbital rotator with speed control
4.3.7   3500 MWCO dialysis membrane
4.3.8   Lyophilizer

4.3.9   2 mL Eppendorf sample tubes
4.3.10 4 °C refrigerator
4.3.11 -20 ℃ freezer
4.3.12 -80 ℃ freezer
4.3.13 Oven that can reach 120 ℃
4.3.14 Single quadrupole gas chromatography/mass spectrometry (GC/MS) system with an
        electron ionization source. The system should have a mass range of 10 u to 1050 Da, a
        scan speed of 10 000 Da/s and mass axis stability ≥ 0.1 Da/48 h
4.3.15 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$_2$O so that the final concentration of DNA in the tube is ≤ 500 µg/mL.
5.1.2   Place the tube on an 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 nm to 30 mm diameter) the DNA against ddH$_2$O 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 [17]. 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 [18] or
        Bradford [19] 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, $\cong$ 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 1 s^{-1} (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 GC/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 GC 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 GC/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 Fpg/Nth buffer to each sample and incubate all samples at room temperature with gentle rocking for ≥ 4 h (do not vortex mix samples). The Fpg/Nth buffer is prepared by adding the following ingredients to ddH$_2$O (50 mmol/L sodium phosphate, 100 mmol/L potassium chloride, 1 mmol/L EDTA, and 0.1 mmol/L DTT) and adjusting the pH to 7.4.

6.1.5   If the lesion levels for 8-OH-Gua are going to be tested, add the internal standard for this lesion at this point. 8-Hydroxyguanine is more readily oxidized than the other internal standards and is thus added at this step instead of in step 6.1.2. The concentration of the 8-OH-Gua internal standard to be added should be determined by UV spectrophotometry before use due to its limited stability.

6.1.6   Add Nth and/or Fpg to the samples. These enzymes are commercially available and can also be prepared using recombinant methodology [20]. The concentration of enzyme(s) to be added should be determined during preliminary experiments by adding a range of concentrations and assessing the optimal concentration for maximum excision of oxidized lesions.

6.1.7   Briefly centrifuge the samples at 1000 g for 30 s at room temperature to ensure that all of the solution is at the bottom of the container.

6.1.8   Incubate the samples at 37 °C for 1 h (water bath).

6.1.9   Stop the reaction by adding 150 µL of absolute cold ethanol and briefly vortex mix each sample. This step will precipitate the unreacted DNA glycosylases and the undigested DNA (DNA containing non-modified bases).

6.1.10 Incubate all samples at -20 °C for 1 h or overnight.

6.1.11 Centrifuge all samples at 15 800 g for 30 min at 4 °C.

6.1.12 Transfer supernatant (185 µL) into clear crimp-top glass autosampler vials. Leave the undigested DNA at the bottom of the Eppendorf tube.

6.1.13 Remove the ethanol from the autosampler vials by placing the vials in the Speed Vac or in a vacuum desiccator. Preliminary experiments can be conducted to find the time needed to fully remove the ethanol.

6.1.14 Add 200 μL of ddH$_2$O to each sample and cover the glass vials using tissue and rubber bands. The tissue prevents the ice from flying out of the vials. Insert the samples into a round glass sample holder and freeze all samples using liquid nitrogen. Pour off the excess liquid nitrogen from the round glass vial holder. Alternatively, the samples can be frozen in a freezer at -80 ° C for at least half an hour. Dry all samples overnight (minimum of 16 h) using a freeze dryer. Freeze drying does not form precipitates, instead a film of DNA should cover the bottom quarter of the vial at the end of the drying step.

6.1.15 Store the samples at room temperature in a glass jar containing fresh desiccant or proceed directly to derivatization which starts in the next step.

6.1.16 Turn on an oven and let it warm up to 120 ° C. Remove BSTFA from the refrigerator at 4 ° C and let it warm up for 10 min at room temperature.

6.1.17 Derivatize analytes to trimethylsilyl esters by adding 30 μL of BSTFA and 30 μL of pyridine to each sample vial.

6.1.18 Cover each sample with a light stream of high purity nitrogen (99.999%) for ≅10 s, close sample vials and vortex for ≅ 5 s. 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. Note: connect an oxygen and moisture removal cartridge to the outlet of the high purity nitrogen supply to ensure the purity of the nitrogen entering the samples.

6.1.19 Incubate samples at 120 °C for 30 min.

6.1.20 Allow samples to cool for approx. 15 min (leave them on a lab bench).

6.1.21 Transfer samples into GC/MS autosampler vials (containing low-volume inserts) with syringe (wash syringe with acetonitrile solution after each sample transfer).

6.1.22 Cover samples with a light stream of nitrogen for ≅10 sec and crimp-cap.

6.1.23 Analyze the samples using GC/MS methodology [21-23].

6.2 GC/MS sample analyses

6.2.1 The GC/MS sample analyses are performed on a high resolution single quadrupole GC/MS system operated in positive ion mode with an electron ionization source.

6.2.2 The GC column is an HP-Ultra 2 high-resolution fused silica capillary column (12.5 m, 0.2 mm i.d.) coated with cross-linked 5 % phenylmethylsilicone gum phase (film thickness, 0.33 μm) (Agilent Technologies).

6.2.3 Gas chromatography separation of the derivatized lesions is performed with a temperature programmed ramp from 130 °C to 300 °C (130 °C, 2 min hold, 8 °C per min to 207 °C, 0 min hold; 10 °C per min to 300 °C, 4 min hold). The total run time is 24.75 min. The sample injection amount is 5 μL.

6.2.4 Trimethylsilyl derivatives of each DNA lesion and their stable isotope-labeled analogue are detected in SIM mode after appropriately tuning and optimizing the 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 derivatized analytes are the following: analyte dwell time, injection mode, split ratio, split flow rate, inlet temperature, inlet pressure, MS source temperature, GC/MS interface temperature, MS quadrupole temperature, MS quadrupole voltage, repeller voltage, extractor voltage, electron energy voltage and MS run time. The relevant SIM ions are:

m/z 354, 368, 369 and *m/z 357, 371,* 372 for FapyAde and FapyAde-^{13}C,^{15}N$_2$, respectively; *m/z 442, 457* and *m/z 445, 460* for FapyGua and FapyGua-^{13}C,^{15}N$_2$, respectively; *m/z 440, 455* and *m/z 445, 460* for 8-OH-Gua and 8-OH-Gua-^{15}N$_5$, respectively; *m/z 352, 367* and *m/z 355, 370* for 8-OH-Ade and 8-OH-Ade-^{13}C,^{15}N$_2$, respectively; *m/z 342* and *m/z 345* for 5-OH-Cyt and 5-OH-Cyt-^{13}C,^{15}N$_2$, respectively; *m/z 329* and *m/z 335* for 5-OH-Ura and 5-OH-Ura-^{13}C$_4$,^{15}N$_2$, respectively; *m/z 343, 358* and *m/z 347, 362* for 5-OH-MeUra and 5-OH-MeUra-^{13}C$_2$, d$_2$, respectively; *m/z 259, 433, 448* and *m/z 262, 437, 452* for ThyGly and ThyGly-d$_4$, respectively, and *m/z 331* and *m/z 334* for 5-OH-5-MeHyd and 5-OH-5-MeHyd-^{13}C,^{15}N$_2$, respectively. [21-23]. The SIM current profiles are integrated using appropriate peak integration software and the measured lesion levels are determined using the SIM 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

5-OH-Cyt	5-hydroxycytosine
5-OH-Ura	5-hydroxyuracil
5-(OHMe)Ura	5-(hydroxymethyl)uracil
5-OH-5-MeHyd	5-hydroxy-5-methylhydantoin
8-OH-Ade	8-hydroxyadenine
8-OH-Gua	8-hydroxyguanine
FapyAde	4,6-diamino-5-formamidopyrimidine
FapyGua	2,6-diamino-4-hydroxy-5-formamidopyrimidine
BSTFA	bis(trimethylsilyl)trifluoroacetamide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ENM	engineered nanomaterial
NP	nanoparticle
SIM	selected-ion-monitoring
SRM	standard reference material
ThyGly	thymine glycol
UV/Vis	ultraviolet-visible

8   Acknowledgements

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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) [24-27]. 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 [28]:

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

(2) $\qquad v_t = \dfrac{d_r}{d_t}$

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

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.
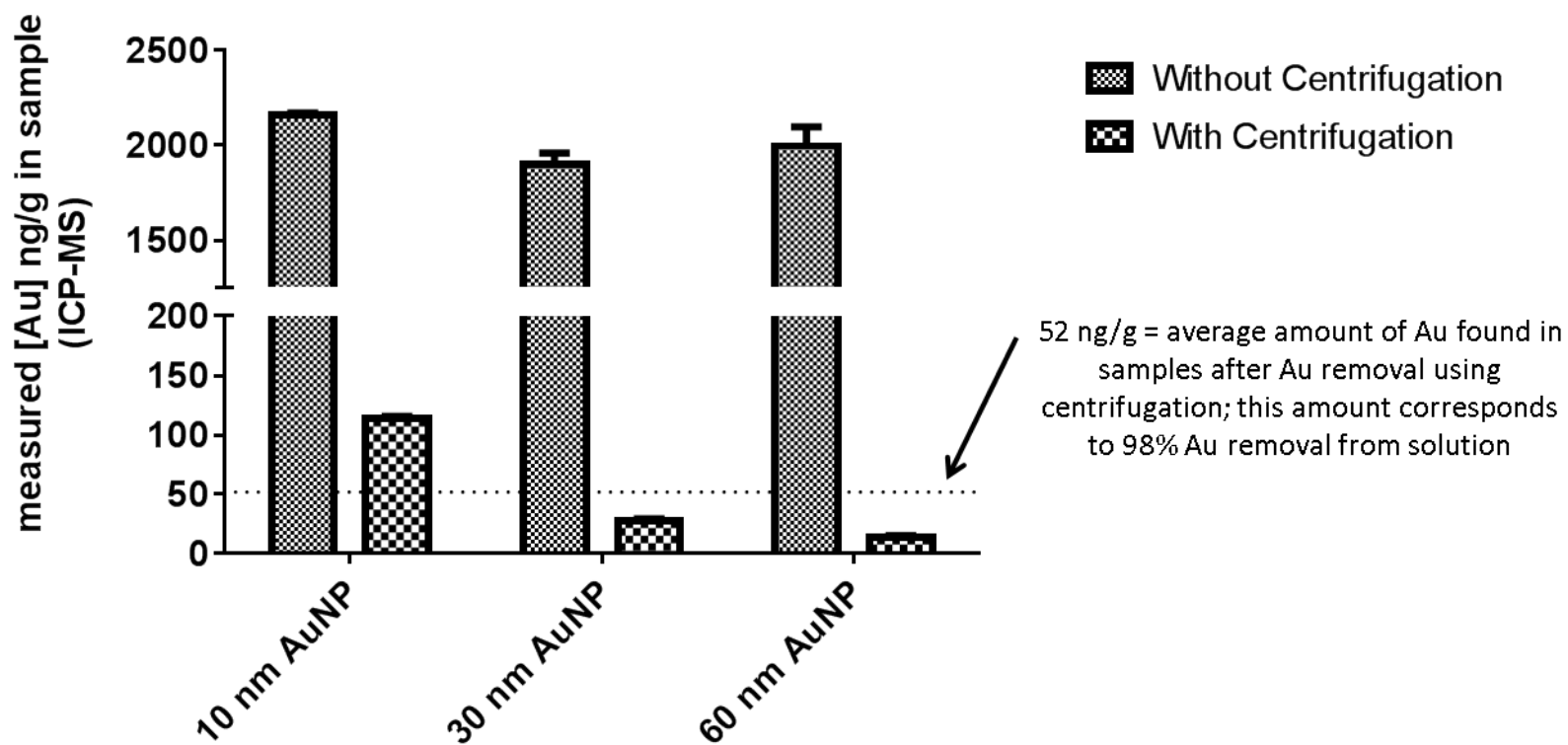
3. Table 1

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 TiO_2 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 %.