GUIDELINES FOR NANOTOXICOLOGY RESEARCHERS USING NANOCOMPOSIX MATERIALS

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Please contact us at info@nanocomposix.com or 858-565-4227 with any questions or suggestions.

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Welcome to our Nanotoxicology Guide. This document is designed to help our customers maximize the impact of their research by providing them with information on a variety of subjects, ranging from storage and handling guidelines to detailed protocols for testing the concentration of silver ions and endotoxins. While this guide is primarily directed towards silver nanoparticles, we hope that you find this information useful for other types of nanoparticles. If you have any questions or comments, please feel free to contact us at info@nanocomposix.com or (858) 565-4227 x 2.

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General Handling Guidelines:

The following section includes Storage, Handling, and Safety guidelines for nanoComposix silver nanoparticle products.

Storage:

Store products away from light at 4-6 ^{o}C in a sealed container. Short periods at room temperature are acceptable, however lower temperature storage prolongs the shelf life of the product.

DO NOT FREEZE. If frozen, the silver nanoparticles will irreversibly aggregate and the solution color will change. When stored at 4^{o}C and away from light, the NanoXact silver nanoparticles are stable for at least 1 year, and BioPure silver nanoparticles are stable for at least 6 months.

Handling:

During storage the silver nanoparticles may settle to the bottom of the vial. Prior to aliquoting or using the nanoparticles, resuspend the settled nanoparticles by vigorously shaking the bottle until a homogenous solution is obtained. Typically this will require approximately 20 seconds of mixing. Visually inspect the bottom of the container to ensure that there are no remaining settled particles.

Safety/MSDS

BioPure and NanoXact Silver Material Safety Data Sheet (MSDS) reports can be found at nanocomposix.com/support/msds.

Experimental Design Guidelines:

A number of recent research studies have demonstrated the effect that nanoparticle size, shape, agglomeration state and residual chemicals can have on nanomaterial toxicology. Because of this relationship, it is critical that experiments account for the state of the nanoparticles as well as residual chemicals (such as silver ions or capping agents) in solution. The following checklist and detailed protocols are designed to help our customers perform the pertinent experimental controls and ensure that their research has the maximum possible impact.

We've identified four control tests that are critical to generating high quality data:

1. Nanoparticle Stability
2. Silver Ion Content Control Testing
3. Residual Reactant and Capping Agent Toxicity Testing
4. Endotoxin Control Testing

Nanoparticle Stability:

Before beginning a new experiment, we recommend researchers confirm that the nanoparticle formulations to be used are stable to ensure that toxicity results are linked to the size and shape of the

nanoparticles being tested. While NanoXact and BioPure formulations are guaranteed to remain stable for 1 year and 6 months, respectively, variations in the storage and handling can result in changes in nanoparticle size or agglomeration state.

UV Visible Spectroscopy

One of the simplest ways to confirm nanoparticle stability is to monitor the UV/Visible optical spectrum of nanoparticle solutions. Because silver nanoparticles support electron oscillations (known as plasmon resonances), they have unique UV/Visible spectra that change as a function of nanoparticle size, shape, and concentration (**Figure 1**). When particles destabilize, their spectral properties begin to change significantly, resulting in an increase in OD at longer wavelengths, and a change in the nanoparticle appearance (**Figures 2 and 3**).

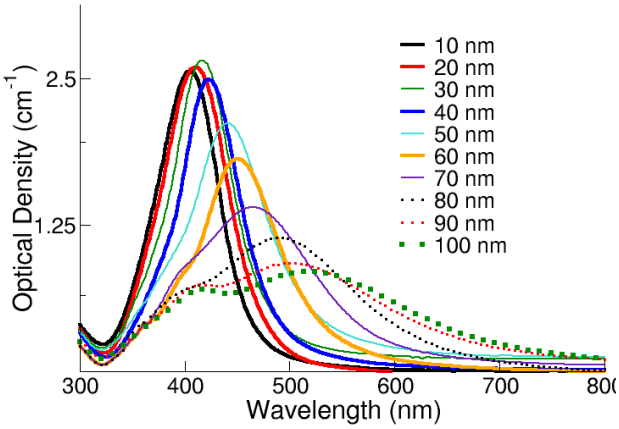

Figure 1: Silver nanoparticle UV/Visible spectra as a function of particle size. Note that the peak optical density (OD) and wavelength strongly depend upon particle size.

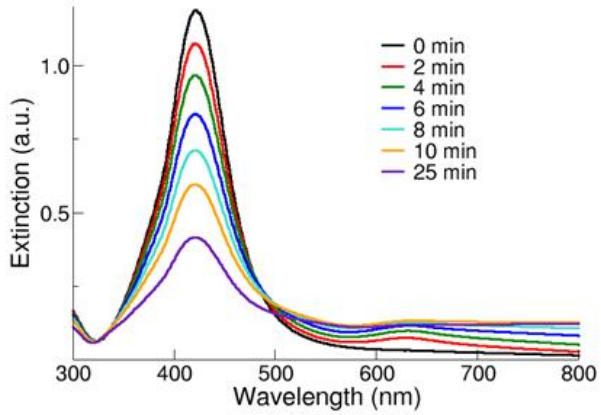

Figure 2: UV/Visible spectra of silver nanoparticles as they destabilize. Note that as they agglomerate, the peak optical density at ~420 nm decreases, while the optical density within the 600 – 800 nm range increases.

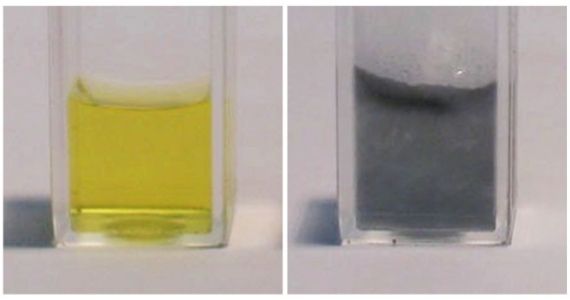

Figure 3: Visual inspection can also be a useful method to monitor nanoparticle stability. Stable silver nanoparticles (left image) appear yellow or opaque white/yellow, while destabilized or agglomerated silver nanoparticles appear grey or as a black precipitate at the bottom of the vial.

Transmission Electron Microscopy

Transmission Electron Microscopy (TEM) is the best method of determining the size and shape of silver nanoparticles but is not so useful for determining agglomeration state. To prepare nanoparticles for imaging on a TEM, the nanoparticles are dried onto a carbon coated copper grid. When the particles dry, capillary drying forces often bring the particles into clusters that make it appear that they are agglomerated even though they were individual when in solution.

Dynamic Light Scattering

Dynamic light scattering monitors the scattering of light from nanoparticles to determine their average size in solution. In contrast to TEM which measures the primary size of the nanoparticles, the DLS measures the hydrodynamic size and is very sensitive to nanoparticle agglomeration. Typically, DLS measurement will yield a slightly larger diameter than the TEM determined size due to capping ligands and the charged double layer surrounding the nanoparticle. However, at very small sizes (<20 nm), the scattering component of nanoparticles is very low and even at high concentration the instrument is not very accurate. For this reason, DLS measurements are not typically provided for particles less than 20 nm in diameter.

Protocol for Testing NanoXact and BioPure Silver Nanoparticle Stability: UV/Visible Spectroscopy

This section describes a method to perform QC checks on NanoXact Silver Nanoparticles. In general, we recommend that a stock check be performed every 3 months, or immediately before performing critical experiments.

Materials:

1. UV/Visible instrument such as those sold by Varian, Ocean Optics, Avantes, Agilent, and many other commercial vendors.
2. Milli-Q or DI water
3. Silver nanoparticle solutions
4. Quartz or methacrylate cuvettes with a 10 mm path length and 4.5 mL capacity.

Methods:

1. **Instrument Setup:** Ensure that instrument lamp(s) are on and warmed up.
2. **Prepare Blank Cuvette:** Pipette 2 mL of Milli-Q or DI water into a clean and dry cuvette.
3. **Collect Blank Spectrum:** Insert the cuvette into the spectrometer, and collect a *Blank* spectrum.
4. Prepare Sample Cuvette:
5. (For NanoXact) Pipette 1 mL of silver nanoparticles into the cuvette containing 2 mL of water used to measure the blank, and pipette the solution in and out of the cuvette 5 times to ensure the water and nanoparticle solutions are well mixed.

6. (For BioPure) Pipette 10 µL of silver nanoparticles into the cuvette containing water used to measure the blank, and pipette the solution in and out of the cuvette 10 times to ensure the water and nanoparticle solutions are well mixed.
7. **Collect Sample Spectrum:** Insert the sample cuvette into the spectrometer and collect a *Sample* spectrum.
8. **Verify Pass/Fail Status:** Compare the measured peak optical density and wavelength of the diluted sample solution to the values in the table below (**Table 1 and 2**). If the values of the diluted solution fall within the listed OD and wavelength ranges for the specified nanoparticle size, then the material has passed QC, and can be considered stable and unagglomerated. If the values do not fall within the specified QC range, please contact a nanoComposix scientist at Service@nanocomposix.com to discuss your data.
9. **Record Data:** Record the UV/Visible spectrum, peak optical density, and wavelength at peak optical density in order to track any small changes that may occur over time. Should the particles begin to destabilize, this data will help nanoComposix scientists diagnose the likely cause of the stability issue.

While the peak optical extinction and the peak wavelength are important metrics for interpreting optical spectra, there are other features in the spectrum that are important. For example, the presence of a secondary peak at a longer wavelength than the primary peak is often an early indication of agglomeration (see **Figure 4**). Overlaying the spectra from the same batch of nanoparticles at different time points is a useful method of understanding changes to the nanoparticles over time.

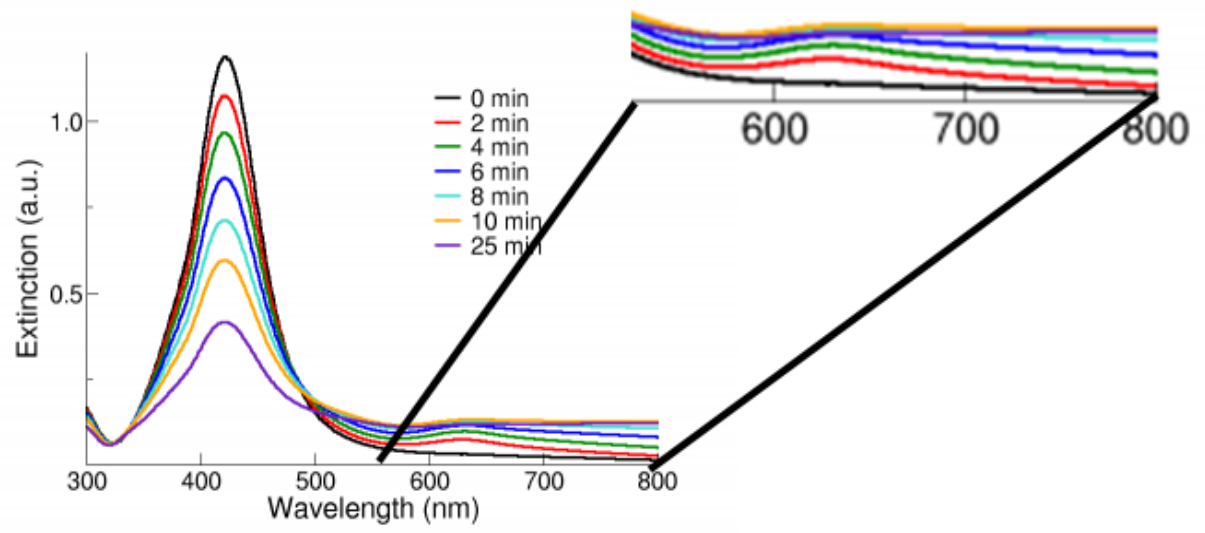

Figure 4: Spectra of silver nanoparticles in the presence of 0.15 M NaCl. Note the formation of a secondary peak between 600 and 700 nm that is indicative of agglomeration.

Nanoparticle Diameter	Peak Optical Density (NanoXact)	Peak Optical Density (BioPure)	Wavelength at Peak
10 nm	0.7 - 0.95	0.52 - 0.71	380 - 405 nm
20 nm	0.7 - 0.95	0.52 - 0.71	390 - 410 nm
30 nm	0.6 - 0.85	0.45 - 0.64	390 - 410 nm
40 nm	0.75 - 1.05	0.55 - 0.78	405 - 425 nm
50 nm	0.7 - 0.95	0.52 - 0.71	410 - 430 nm
60 nm	0.55 - 0.75	0.41 - 0.55	425 - 450 nm
70 nm	0.45 - 0.65	0.33 - 0.49	430 - 455 nm
80 nm	0.4 - 0.55	0.29 - 0.41	440 - 480 nm
90 nm	0.35 - 0.5	0.26 - 0.38	470 - 510 nm
100 nm	0.25 - 0.35	0.186 - 0.26	480 - 530 nm
110 nm	0.21 - 0.29	0.15 - 0.22	485 - 550 nm

Table 1: Peak optical density (cm^{-1}) and wavelengths for NanoXact Silver Nanoparticles characterized using the protocol described above. Please note that the listed optical density values are for samples that have been diluted rather than for the stock solutions. Refer to **Table 2** for the Optical Density (cm^{-1}) for undiluted NanoXact and BioPure silver nanoparticles.

Nanoparticle Diameter	Peak Optical Density (NanoXact)	Peak Optical Density (BioPure)	Wavelength at Peak
10 nm	2.1 - 2.85	104.5 - 142.7	380 - 405 nm
20 nm	2.1 - 2.85	104.5 - 142.7	390 - 410 nm
30 nm	1.8 - 2.55	90.5 - 128.6	390 - 410 nm
40 nm	2.25 - 3.15	110.5 - 156.8	405 - 425 nm
50 nm	2.1 - 2.85	104.5 - 142.7	410 - 430 nm
60 nm	1.65 - 2.25	82.4 - 110.5	425 - 450 nm
70 nm	1.35 - 1.95	66.3 - 98.5	430 - 455 nm
80 nm	1.2 - 1.65	58.3 - 82.4	440 - 480 nm
90 nm	1.05 - 1.5	52.25 - 76.4	470 - 510 nm
100 nm	0.75 - 1.05	37.4 - 52.25	480 - 530 nm
110 nm	0.63 - 0.85	31.5 - 42.5	485 - 550 nm

Table 2: Peak Optical density (cm^{-1}) and wavelengths for undiluted NanoXact and BioPure Silver Nanoparticles. Please note that most spectrometers are unable to operate effectively with high OD solutions, which is why we recommend diluting samples as described in the protocol above.

Silver Ion Content Testing

Results from a number of recent journal articles suggest that silver ions, rather than the zero valent silver nanoparticles are the primary source of toxicity that has been attributed to silver nanoparticles. The release rate of silver ions from a suspension of silver nanoparticles is dependent on the surface area of the nanoparticles, the temperature, water oxygen content, and the presence and identify of surface bound ligands on the nanoparticle surface. The lifetime of free silver ions in solution also depends on the suspension environment with light exposure and salt concentration being important variables. Due to the linear relationship between the surface area to volume ratio for a fixed concentration of silver nanoparticles, smaller nanoparticles will have a higher release potential (see **Table 3**). Also, smaller nanoparticles have a higher rate of curvature and are more likely to release a silver molecule from the surface. This effect of smaller particles dissolving at a higher rate than larger particles is referred to as Ostwald ripening and is an important effect for nanoparticles with diameters less than 10 nm.

The standard method of testing the concentration of ionic silver measures the concentration of silver ions in solution after removing the silver nanoparticles. To isolate the solution, we recommend using a centriprep centrifuge filter or high speed centrifugation to pellet the nanoparticles, followed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) to determine the silver ion concentration of the supernatant solution. The following table includes suggestions for centrifugation speeds and durations that will efficiently separate the supernatant from the nanoparticle pellet in our standard NanoXact and BioPure products.

Nanosphere Diameter (nm)	Surface area (nm^2)	Volume (nm^3)	Ratio Surface Area to Volume
2	13	4	3.0
5	79	65	1.2
10	310	520	0.6
20	1,300	4,200	0.3
50	7,900	65,000	0.12
100	31,000	520,000	0.06
200	130,000	4,200,000	0.03

Table 3: Surface area, volume, and ratio of surface area to volume of nanospheres of varying diameter.

Protocol for Testing Silver Ion Concentration in NanoXact and BioPure Nanoparticle Formulations

1. Pipette 1 mL of nanoparticle solution into 1.5-2 mL conical bottom microfuge tube, and cap the tube (**Figure 5**).
2. Place tubes in centrifuge rotor and spin the samples at the speed and duration listed in **Table 4**. Note that for particles < 30 nm, a standard centrifuge may not supply the necessary force and an ultracentrifuge may be necessary. Centriprep filtration devices with a low MW cut-off may also be used to isolate the nanoparticles from solution.

Figure 5: NanoXact silver nanoparticles in 1.5 mL conical bottom microfuge tube.

Figure 6: Silver nanoparticles in 1.5 mL conical bottom microfuge tube.

Figure 7: A pipette is inserted 1-2 mm from the nanoparticle pellet as the supernatant is gently removed.

3. Gently remove the sample from the centrifuge. The nanoparticles will have formed an "oil" or "pellet" at the bottom of the tube and the supernatant should be completely clear (**Figure 6**).
4. Using a pipette, remove the supernatant from the microfuge tube (**Figure 7**). Gently place the pipette tip within 1-2 mm of the nanoparticle oil, and remove the liquid as gently as possible with minimal disturbance of the nanoparticle oil.
5. Use the supernatant for ICP-MS testing following the instructions for the instrument you are using.

Nanoparticle Diameter	Centrifugation Speed (rcf)	Duration (min)
10 nm	40000	120
20 nm	25000	90
30 nm	20000	60
40 nm	16000	45
50 nm	15000	30
60 nm	14000	30
80 nm	12000	30
100 nm	9000	30

Table 4: This table lists the speed and duration required to remove silver nanoparticles from buffer solution for our silver nanoparticle products. Please note that in general this is considered a destructive test, and the nanoparticles will not be viable after being centrifuged into a pellet at these speeds.

Effect of Residual Reactants and Stabilizers on Toxicity Assays

During nanoparticle fabrication various chemical reagents are used to formulate and stabilize nanoparticles. Some of these reagents can impact subsequent toxicology experiments and it is important to isolate the effect of chemical reagents in solution from the nanoparticles. At nanoComposix, we extensively wash and purify our OECD and BioPure line of nanoparticles. This is accomplished using a proprietary filtration step that allows for 10 volume washes of the nanoparticle solution reducing the concentration of molecular ion species by more than 99.99%. However, for molecules with a molecular weight similar or larger than the pore cut-off size of the filter, these materials will be retained and potentially concentrated along with the nanoparticles. For nanoparticles such as silver and gold that have high material densities, centrifugation is an effective method for separating the nanoparticles from residual molecular species. Even though there has been no experimental evidence that any residual materials other than released silver ions are resulting in a toxic response from our Biopure materials, this is an important control and we recommend its inclusion in toxicology experiments when using nanoComposix's materials or materials from other sources.

Protocol for Testing Residual Chemical Toxicity in NanoXact and BioPure Nanoparticle Formulations

1. Follow Steps 1 - 4 in Protocol for Testing Silver Ion Concentration in NanoXact and BioPure Nanoparticle Formulations above.
2. From one sample, measure the metal ion concentration using ICP-MS.
3. From another sample, use the supernatant as a control solution in your toxicology experiments
4. Prepare a metal ion solution at the same silver ion concentration as was measured with the ICP-MS using a metal salt (e.g. silver nitrate). Include this metal ion solution in your toxicology experiments.
5. Compare the end point results from the supernatant and the metal ion solution to determine if there is any additional toxicity associated with other materials that are in the nanoparticle solution.

Endotoxin Testing

Another critical control test is to determine the endotoxin concentration in nanoparticle solutions. Endotoxins are components of the outer membranes of bacteria, most commonly gram negative bacteria, that are released when cells are disrupted. When the concentration is high, they can produce a toxic response. Endotoxin contamination can come from the reagent materials used in nanoparticle fabrication or be introduced during subsequent fabrication and processing steps. At nanoComposix, all BioPure nanoparticle formulations are prepared using aseptic protocols and each starting reagent is tested to ensure they contain extremely low endotoxin levels. Using synthesis reagents with low endotoxin concentration is critical since, due to their larger size, endotoxins will be concentrated along with the nanoparticles during the filtration concentration step.

Silver nanoparticles have unusual size, shape, and agglomeration dependent optical properties that interfere with standard endotoxin tests. Initial attempts to use a chromogenic endotoxin assay failed

due to the strong optical properties of silver nanoparticles. The chromogenic endotoxin assay utilizes a synthetic chromophore substrate that becomes strongly colored when exposed to endotoxins. The color intensity is linear with respect to endotoxin concentration over a wide range, allowing the endotoxin concentration to be calculated through comparison with a standard curve. While this is commonly considered the simplest of endotoxin tests and is highly sensitive, with a lowest limit of detection (LLoD) of ~0.005 EU/mL, the chromogenic color intensity is measured at 405 nm, which overlaps with the silver nanoparticle extinction peaks (**Figure 1**). Due to the large optical response of the silver nanoparticle solutions, the additional colorimetric signal from the assay is not accurately measured and results in erroneous values.

In 2011, we modified a test developed by the National Characterization Laboratory (NCL) at the National Cancer Institute (NCI) that accurately measures endotoxin levels in the presence of silver nanoparticle solutions using a Pyros Kinetic Turbidity Assay Instrument. As of 2012, all BioPure solutions sold by nanoComposix are guaranteed to have an endotoxin concentration of less than 2.5 EU/mL, a threshold selected in collaboration with the NCL. This corresponds to an endotoxin mass concentration of < 0.3 ng/mL for 1 mg/mL solution of silver nanoparticles. We believe that the Kinetic Turbidity Assay is the most accurate method for measuring endotoxin levels in the presence of silver nanoparticles. However, there are some important modifications to the standard procedure that need to be made in order to obtain accurate results and these are discussed below.

Overview of the Kinetic Turbidity Assay

The Kinetic Turbidity Assay is based upon the reaction between endotoxin and a lysate. If endotoxin is present, the lysate reagent causes a clotting reaction which increases the solution optical density. A spectrometer measures the OD at 660 nm, as a function of time, and monitors the time required to reach 20 mAbs (0.2 OD higher than the initial sample baseline as time= zero, **Figure 8**). This data is compared to a standard curve to quantitatively calculate the endotoxin concentration.

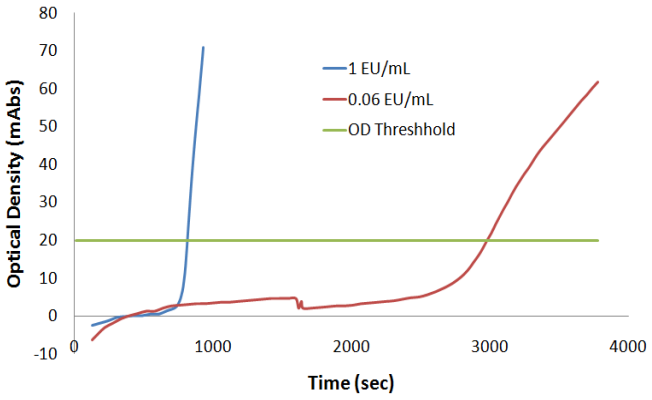

Figure 8: OD vs. time data collected in a Kinetic Turbidity assay.

The assay is highly sensitive, with a lowest limit of detection of ~0.005 EU/mL, and has been verified by the NCI's Nanotechnology Characterization Laboratory and nanoComposix to work extremely well with nanoparticle formulations. Two excellent resources to learn more about endotoxin assays that can be used for nanomaterials can be found at the NCL's website as well as in this journal article.

Protocol for Testing Endotoxin Concentration in BioPure Nanoparticle Formulations

The protocol for the Kinetic Turbidity LAL Assay essentially follows the procedure outlined in NCL Method STE-1.2 that is available online. The main challenge in adapting this procedure to the testing of

silver nanoparticles is the change in the optical properties of the silver nanoparticles due to destabilization of high concentrations of silver nanoparticles in the presence of the KT reagents. This can result in the two following issues:

Baseline OD issues: The assay detects OD at 660 nm. Larger silver nanoparticle formulations have significant extinction at 660 nm. If the background OD is relatively high (e.g. >0.1 or 0.2), then this can reduce the dynamic range of the assay. For samples with high OD at 660 nm, the sample must be diluted to reduce the baseline OD, or the supernatant must be tested in the absence of nanoparticles.

Changing baseline issues: This assay typically collects sample baseline OD data for the 1st 30 sec-5 minutes (this is controlled by the user). If the lysate causes the particles to aggregate, this can significantly increase the baseline OD at 660 nm (**Figure 4**), which can result in a false positive detection of endotoxin. The solution is to reduce the duration of the baseline collection. If the base collection reduction is still not sufficient to obtain accurate results, the sample concentration needs to be reduced to lower the impact on the baseline.

To avoid both Baseline and Changing Baseline issues, we recommend that nanoComposix BioPure materials be tested using the dilutions listed in **Table 5**. Alternatively, a higher limit of detection can be obtained by spinning out the silver nanoparticles and measuring the concentration of endotoxin in the supernatant. In internal tests at nanoComposix, more than 85% of the measured endotoxin content remains in the solution compared to that bound to the nanoparticle surface. We also provide endotoxin testing of each batch of BioPure nanoparticles, and provide this information to our customers in the specification sheets provided with each order.

Nanoparticle Diameter	Suggested Dilution of BioPure Formulations
10 nm	1:500
20 nm	1:250
30 nm	1:150
40 nm	1:100
50 nm	1:100
60 nm	1:100
80 nm	1:50
100 nm	1:50

Table 5: Suggested dilutions for BioPure formulations using Kinetic Turbidity Assay. NanoXact formulations can be tested without dilution.

ADDITIONAL USEFUL INFORMATION

The following sections describe useful information and tips that are pertinent for nanotoxicology researchers.

- Preparation of stock solutions
- The effect of salt on nanoparticle stability, and order of addition of buffer components
- Monitor stability and aggregation state after buffer incubation and throughout the experiment
- TEM Imaging

Preparation of Stock Solutions

Biopure silver nanoparticle solutions are provided at a concentration of 1 mg/mL and can be diluted. Nanoparticles coated with PVP or tannic acid can be diluted directly into DI water however citrate, which is a highly displaceable capping agent, needs to be diluted into a citrate buffer (2mM). Nanoparticle suspensions are very sensitive and researchers should be very careful not to destabilize the material by exposing to a dirty pipette tip or container. Pouring from the received bottle is always the best way of transferring solutions to a new container.

The effect of salt on Nanoparticle stability, and order of addition of buffer components

The surface of most nanoparticles is dynamic and is strongly influenced by the local environment. Different suspension conditions can have dramatic effects on the charge and stability of the nanoparticle. High salt environments will collapse the charged double layer that surrounds suspended nanoparticles and can result in nanoparticle aggregation. Proteins and other biomolecules will often associate with and stabilize particles. When working with nanoparticles you must anticipate changes to the surface and plan accordingly.

For example, when nanoparticles are to be added to solutions used in in-vitro and in-vivo experiments, it is best to expose the particles to a low-salt solution that contains the protein components of the media and then add salt to bring the solution up to isotonic conditions. The protein binds to the particle surface and the suspension stability increases. If the nanoparticles are added directly to a high salt buffer, the particles may agglomerate before protein stabilization can occur.

Monitor stability and aggregation state after buffer incubation and throughout the experiment

When nanoparticles are exposed to high salt solutions such as those present in most biologically compatible media, agglomeration can occur. Depending on the nanoparticle material, size, and surface, the agglomeration can happen instantaneously or over a period of days. Once the particles agglomerate they behave like much larger particles and can have rapid settling rates which complicates the measurement of dose and can significantly change toxicity results.

It is critical to understand the rate of agglomeration of nanoparticles in the target system. Due to the presence of cells or other biological or environmental material, it is often not possible to directly monitor the agglomeration state once the nanoparticles are delivered. Therefore, it is highly recommended that stability studies be performed in a particulate free medium that is a close mimic to the target environment. Monitoring the UV-Visible spectrum or DLS hydrodynamic diameter over time will provide information on the rate of agglomeration. If the rate is rapid, then additional care must be taken to ensure that the timing associated with dosing events is carefully managed in order to make sure that the nanoparticles are in a reproducible agglomeration state when introduced into the experiment.

TEM Imaging Grid Preparation

At nanoComposix, we use formvar coated copper TEM grids, although almost any formvar coated, carbon coated, or nylon, or SiN grids will be effective. We suggest preparing TEM grids using the NCL protocol or by spotting 2-4 µL of nanoparticle solution on a TEM grid, and allowing it to dry overnight.

Please note that using TEM grids which have been functionalized with amines, thiols, or other reactive chemical can cause the nanoparticle size to change during grid preparation. This has been independently verified by researchers at the University of Oregon, and the data has been recently been published in the journal ACS Nano.

We hope you find this document useful. If you have any questions, or suggestions for additional information or protocols that should be included here, please contact us at info@nanocomposix.com or at (858) 565-4227 x2.

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