Elliott et al.:

Toward Achieving Harmonization in a Nano-cytotoxicity Assay Measurement through an Interlaboratory Comparison Study[1]

Supplemental Protocol S1 – MTS cell viability assay with A549 (serum free treatment)

Attention: Procedures marked with *) can also be implemented with an equivalent other procedure. But it is important to know, what has been done by which institution.

Introduction

It is assumed that each laboratory has its own procedure and protocols to assess and control the cell health and growth.

MTS assay is a colorimetric assay for measuring the activity of enzymes that reduce MTS dyes to formazan dyes, giving a purple color. A main application allows assessing the viability (cell counting) and the proliferation of cells (cell culture assays). It can also be used to determine cytotoxicity of potential medicinal agents and toxic materials, since those agents would stimulate or inhibit cell viability and growth.

Equipment & Reagents

Equipment

- Multiple well plate reader (absorbance)
- Microscope for cell counting
- Hemocytometer
- Centrifuge
- Flat bottom 96 multi-well cell culture plates for MTS absorbance measurements
- Multichannel pipette (at least 8 positions) with 200 µl volume/pipette

Reagents

- Roswell Park Memorial Institute medium (RPMI-1640)
- Fetal Calf Serum (FCS)
- Penicillin 1)
- Streptomycin 1)
- L-glutamine
- 0.05% Trypsin-EDTA
- PBS (Ca and Mg free)

[1] http://dx.doi.org/10.14573/altex.1605021p

- RPMI-1640 – phenol red free
- MTS – CAS number: 138169-43-4
- $CdSO_4$ (10 mM stock solution provided by Empa)
- Trypan blue*) - CAS number: 72-57-1

1) bought as a ready made mixture PenStr from e.g. Gibco

Reagent Preparation

- Complete cell culture medium:
  - Roswell Park Memorial Institute medium (RPMI-1640) with added:
  - 10% FCS (Fetal Calf Serum)
  - 1 x PenStr ready made mixture with:
    - 10 µg/mL Penicilin
    - 10 µg/mL Streptomycin
  - 0.2 mg/mL L-glutamine.

  This media is termed complete cell culture media for the purpose of this SOP.

  Note: Due to L-glutamine potential degradation, cell culture medium must not be stored over more than 3 weeks.

- Cell culture medium without Serum:
  - Roswell Park Memorial Institute medium (RPMI-1640) with added:
  - 1 x PenStr ready made mixture with:
    - 10 µg/mL Penicilin
    - 10 µg/mL Streptomycin
  - 0.2 mg/mL L-glutamine.

  This media is termed cell culture media without Serum for the purpose of this SOP.

  Note: Due to L-glutamine potential degradation, cell culture medium must not be stored over more than 3 weeks.

- $CdSO_4$ Stock Solution: Concentration: 10 mM (stock solution = 100 x working solution) provided by Empa. This stock solution was produced in the following way:
  - 102.64 mg $CdSO_4$ is weighed
  - Transferred in a sterile 50 ml tube
  - Dissolved with 40 ml distilled water
  - --> 40 ml 10mM $CdSO_4$ Store at 4°C (sterile filtration is not necessary)

Cell Culture

All cell cultures are maintained in 75 cm^2 cell culture flasks, in which the cells are passaged at 70-80% confluency every 2-3 days. A549 cells are cultured in complete RPMI-1640 cell culture medium (see Reagent Preparation section) at 37°C, 5% CO_2 in humidified air.

Grow A549-A and A549-B cells (note passage on arrival), which were distributed in frozen form. Upon thawing, cells should be passaged at least 2 times before use in experiments. The cells should not be kept longer than 3 month in culture.

Aliquots of early-passage cells should be frozen down and stored in liquid nitrogen as stock aliquots for later usage.
Note: Immediately after thawing and within the first passage, cell growth may be slow.

A549 cells grow rapidly with a typical population doubling time of 24 h. When the cells are grown in phenol red-containing medium, a change in color from red to yellow could indicate culture overgrowth (i.e. depletion of nutrients). Trypsin is used to remove the cells.

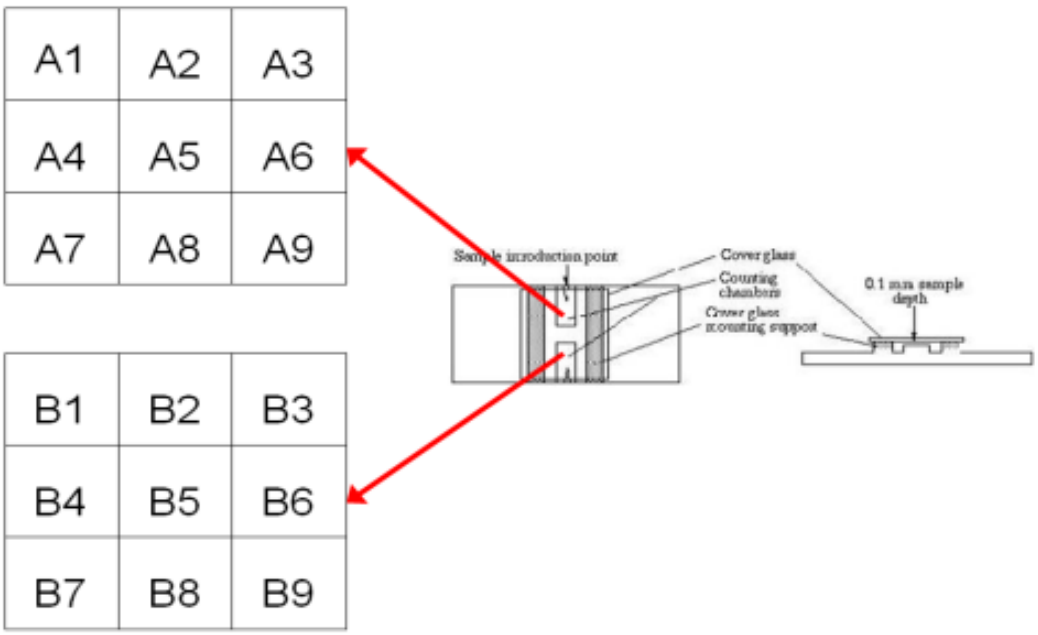
Cell Harvesting

- Cells are grown with 20 mL of complete cell culture media in an incubator under standard conditions (37°C, 5% CO_2 in humidified air).
- Cells are harvest from the cell culture flask by:
    - Wash 2 x the cell culture with 20 mL of PBS
    - Add 2 mL Trypsin-EDTA to the cell culture for 3 minutes
    - Release the cells by vigorous shaking
    - Transfer the cells by adding additional 18 mL of the complete cell culture media by washing the bottom of the cell culture flask several times (3 to 5 times)
    - Count the cells and monitor doubling rate

Counting Cells

Note: A laboratory can use their established counting method for cells. As long as they have shown that the employed method is equivalently performing than the method outlined afterwards.

1. Harvest cells from cell culture media
2. Sample ~ 20 mL (2 mL Trypsin-EDTA + 18 mL of the complete cell culture media) of cell suspension into 50 mL conical tube using a pipette.
3. Centrifuge cell suspension to precipitate cells as pellet for 5 min at $200\times g = 200\times 9.81\mathrm{N}$.
4. Remove the supernatant and add ~ 1 mL media solution and re-suspend the pellet into the media solution
5. Take 30 μL of cell suspension and add into 30 μL of 0.4% (w/v) trypan blue/PBS in a 2 mL microcentrifuge tube. Mix the solution by pipetting with 200 μL micropipette.
6. As live cells can also be stained by trypan blue after extended period of time, perform cell counting in 30 minutes.
7. With a cover-slip in place, use a 200 μL micropipette to transfer 10 μL of trypan blue-cell suspension at each end of the hemocytometer. Try to avoid bubbles within the hemocytometer and cover-slip.



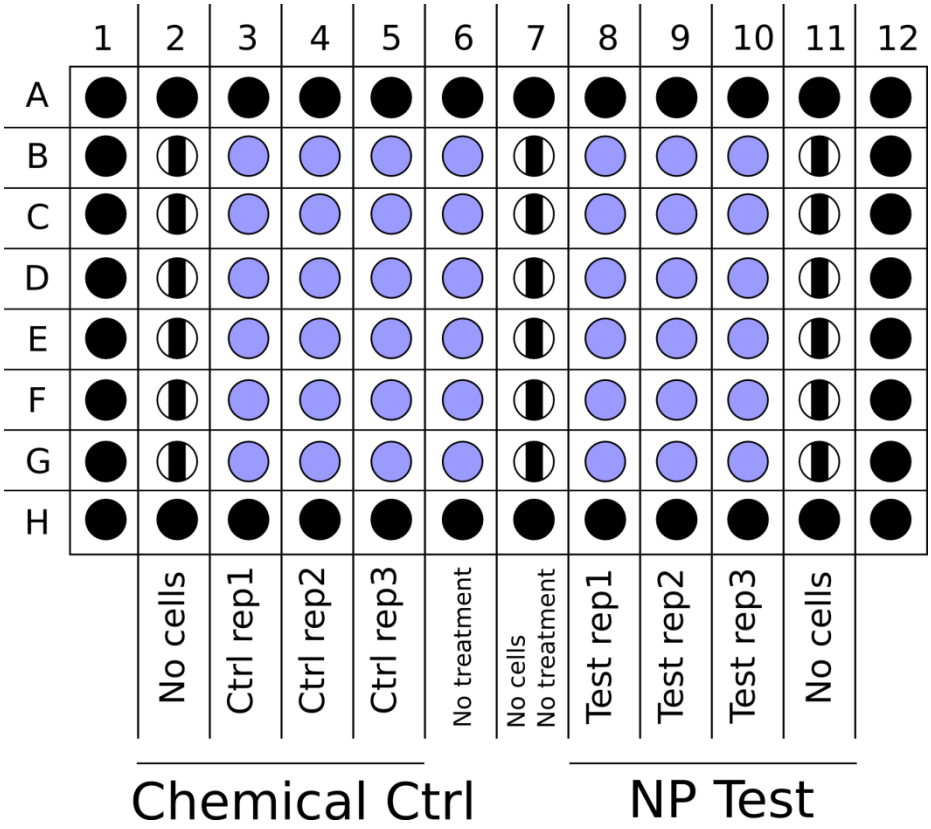
Supplemental Protocol S1, Figure 1: *Schematic diagram of a hemocytometer (cell counting chamber)*

8. Count all the cells (non-viable cells stain blue, viable cells will remain opaque) in the four 1mm^2 squares at the corners (A1, A3, A7, A9, B1, B3, B7 and B9 in Fig. 1), which gives total of eight squares for counting. If greater than 25% of cells are non-viable, the culture is not being maintained on the appropriate amount of media. The current cell suspension should be abandoned and cells have to be recultured.
9. If there are less than 50 or more than 200 cells per large square, repeat the procedure adjusting to an appropriate dilution factor.
10. Calculate the number of cells per unit volume (cells/mL) using the following equation.
    Number of Cells = Average cell count x the dilution factor x 10^4/mL
11. As an example, we could obtain s/m (standard deviation/average cell count) as 3.0/88.6 = 3.4 % from 6 independent sampling and counting (step 7 & step 8) in recent experiment. (Note: These numbers are not a specification. We might be able to specify them at the end of our interlaboratory comparison)

Procedure

Cell Seeding (24 h)

- 7.5x10^5 cells (7.5x10^4 cells/ml) are suspended in 10 mL of complete cell culture medium for one 96 well plate.
- Cells are seeded at 1.5x10^4 cells/well (200µL) in each of the blue wells (see Figure 2) columns 3 – 6 and 8 – 10). It is important that cells in a single column are seeded with a single multichannel pipetting step.
- Stripped wells contain complete cell culture media only.
- Black wells are whenever possible filled with complete cell culture medium only. If operationally necessary they can be kept empty.



Supplemental Protocol S1, Figure 2: *Seeding of the cells in a 96 well plate – Cells are seeded at 1.5x10^4 cells/well with 200 µL complete cell culture medium in each of the blue wells. Stripped wells contain complete cell culture medium only, whereas black wells could either be filled with complete cell culture medium (better) or stay empty. Cells in a single column should be seeded with a single multichannel pipetting step*

- The 96 well plate with the seeded cells is cultured for 24 h in an humidified incubator at 37° C with 5% CO_2

Preparation of the Working Concentrations of the Chemical Control

1. 240 µL sterile H_2O (bi distilled water) are mixed with 23.76 mL cell culture medium without serum. This mixture (1) is used in all the following steps for the preparation of the working concentrations of the chemical controls.

2. 40 µL of 10 mmol/L $CdSO_4$ are mixed with 3960 µL of (1) => 100 µmol/L $CdSO_4$
3. 2000 µL of 100 µmol/L $CdSO_4$ are mixed with 2000 µL of (1) => 50 µmol/L $CdSO_4$
4. 2000 µL of 50 µmol/L $CdSO_4$ are mixed with 2000 µL of (1) => 25 µmol/L $CdSO_4$
5. 2000 µL of 25 µmolL $CdSO_4$ are mixed with 3000 µL of (1) => 10 µmol/L $CdSO_4$
6. 200 µL of 10 µmol/L $CdSO_4$ are mixed with 1800 µL of (1) => 1 µmol/L $CdSO_4$
7. Mixture (1) is used as 0 µmol/L $CdSO_4$

- 10 mmol/L $CdSO_4$ stock solution has been provided by Empa
- The resulting mixtures are vortexed before used for further dilution steps

Note: The previously prepared working concentrations of the chemical controls are sufficient for the treatment of one 96 well plate. If more than one 96 well plates are treated at the same time, then increase the corresponding amounts with the factor of the number of the treated plates, but keep otherwise the ratios the same.
For example: two 96 well plates:
2x40 µL of 10 mmol/L $CdSO_4$ are mixed with 2x3960 µL of (1) => 100 µmol/L $CdSO_4$:=
80 µL of 10 mmol/L $CdSO_4$ are mixed with 7920 µL of (1) => 100 µmol/L $CdSO_4$)

Preparation of the Working Concentrations of the Nanoparticles

1. Cell culture medium without serum is directly used without any addition of water. This medium (2) is used in all the following steps for the preparation of the working concentrations of the nanoparticles.
2. 4 µL of 10% PS-NH_2 are mixed with 3996 µL of (2) => 100 µg/mL PS-NH_2
3. 2000 µL of 100 µg/mL PS-NH_2 are mixed with 2000 µL of (2) => 50 µg/mL PS-NH_2
4. 2000 µL of 50 µg/mL PS-NH_2 are mixed with 2000 µL of (2) => 25 µg/mL PS-NH_2
5. 2000 µL of 25 µg/mL PS-NH_2 are mixed with 3000 µL of (2) => 10 µg/mL PS-NH_2
6. 200 µL of 10 µg/mL PS-NH_2 are mixed with 1800 µL of (2) => 1 µg/mL PS-NH_2
7. Medium (2) is used as 0 µg/mL PS-NH_2

- 10% (w/w) PS-NH_2 stock solution has been provided by Empa
- The resulting mixtures are vortexed before used for further dilution steps and again vortexed before they are used for treatment

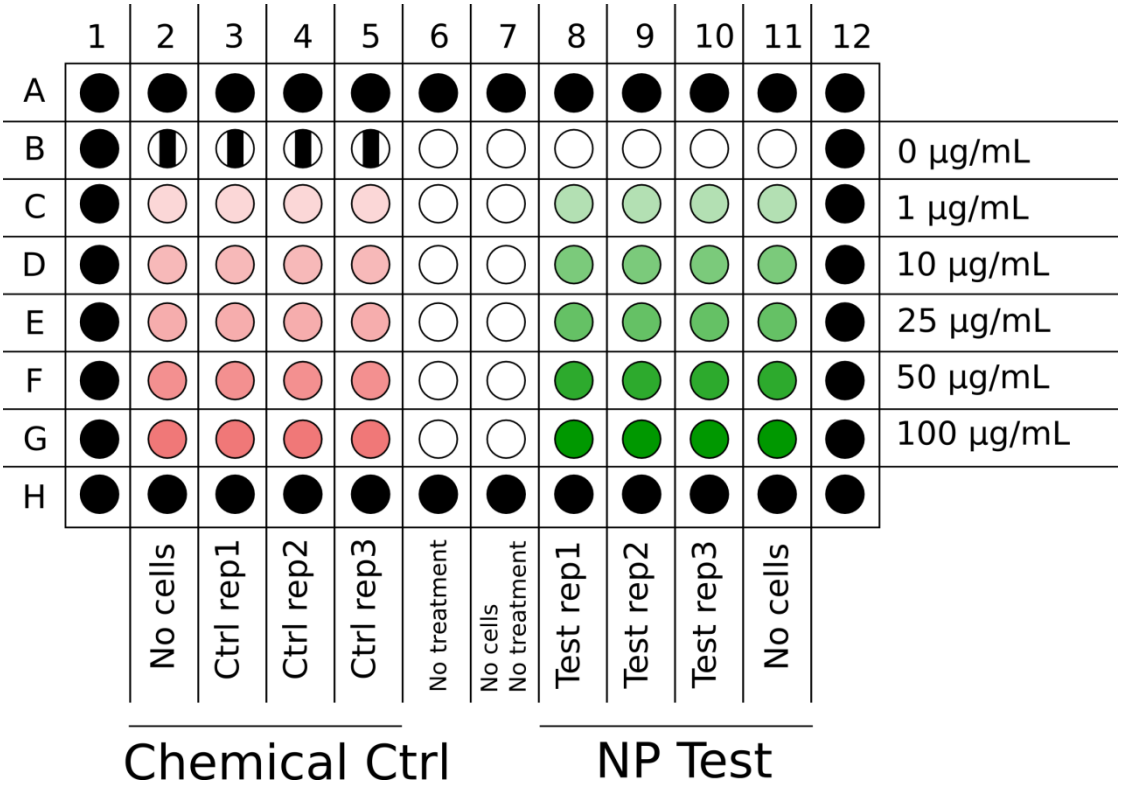
Note:

- The previous preparation of working concentrations of the nanoparticle suspensions are sufficient for the treatment of one 96 well plate. If more than one 96 well plates are treated at the same time then increase the corresponding amounts with the factor of the number of the treated plates, but keep otherwise the ratios the same.
  For example: two 96 well plates:
  2x4 µL of 10% PS-NH_2 are mixed with 2x3996 µL of (2) => 100 µg/mL PS-NH_2 or
  8 µL of 10% PS-NH_2 are mixed with 7992 µL of (2) => 100 µg/mL PS-NH_2
- It is important to record the preparation of the dilution steps in detail. NIST suggests preparing dilutions by putting the tube with the solvent onto a vortexer and then vortexing it while adding the NP containing solution as described in Zook et al (*Nanotoxicology,* 2011, **5**, 517-530).

Treatment of the Seeded Cells with NP and Chemical Control

- Working concentrations of the chemical control (see Preparation of the Working Concentration of the Chemical Control section) and of the nanoparticle (see Preparation of the Working Concentration of the Nanoparticles section) have to be ready for direct transfer into a micropipette (200 µL) before the following steps are performed.
- **Attention:** All working concentrations of the nanoparticles have to be vortexed directly before the transfer into the micropipette.

1. Wells of columns 2 – 5 and of columns 8 – 11 are emptied completely by pumping out the supernatant cell culture medium.
2. **3 x washing with PBS:** Wells of columns 2 – 5 and of columns 8 – 11 are filled with 200 µL PBS per well and then emptied completely. This washing step is repeated three times.
3. Columns 8 – 11 are treated with the working concentrations of the nanoparticles according to the plate layout shown in figure 3.
4. Columns 2 – 5 are treated with the working concentration of the chemical control according to the plate layout shown in figure 3.
5. The 96 well plate is culture for **24 h** after the treatment in a humidified incubator at 37° C with 5% CO_2.



Supplemental Protocol S1, Figure 3: *Dosing plate layout: white wells contain complete cell culture medium, whereas black wells can either contain complete cell culture medium (better) or stay empty. Stripped wells contain mixture (1). Columns 8 – 11 and 2 – 5 are treated column by column with a 10-channel multichannel pipette (200 µL)*

MTS Cell (viability) Activity Assay

Cellular viability is determined by the MTS assay, which measures the reduction of {3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium} (MTS) in the presence of phenazine methosulfate (PMS). It produces a formazan product that has an absorbance maximum at 490-500 nm. After incubation with the indicated doses of NP for 24 h at 37°C, formazan absorbance is measured at 490 nm.

1. 2.5 mL MTS reagents are mixed with 12.5 mL RPMI-1640 medium (phenol red free). This mixture is called (3). There is no addition of serum, L-glutamine, Penicillin and Streptomycin to the (phenol red free) RPMI-1640 medium.
2. All 96 wells of the plate are emptied completely from the supernatant cell culture medium by pumping.
3. All 96 wells of the plate are filled with 120 µL/well of mixture (3).
4. The 96 well plate is incubated for 60 minutes in an humidified incubator at 37° C with 5% CO_2
5. The absorption measurements are performed with a plate reader at a wavelength of 490 nm.
6. These raw absorption results are sent to Empa for the detailed evaluation of the interlaboratory comparison.

Supplemental Protocol S2 – MTS cell viability assay with A549 (serum treatment)

Attention: Procedures marked with *) can also be implemented with an equivalent other procedure. But it is important to know, what has been done by which institution.

Introduction

It is assumed that each laboratory has its own procedure and protocols to assess and control the cell health and growth.

MTS assay is a colorimetric assay for measuring the activity of enzymes that reduce MTS dyes to formazan dyes, giving a purple color. A main application allows assessing the viability (cell counting) and the proliferation of cells (cell culture assays). It can also be used to determine cytotoxicity of potential medicinal agents and toxic materials, since those agents would stimulate or inhibit cell viability and growth.

Equipment & Reagents

Equipment

- Multiple well plate reader (absorbance)
- Microscope for cell counting
- Hemocytometer
- Centrifuge
- Flat bottom 96 multi-well cell culture plates for MTS absorbance measurements
- Multichannel pipette (at least 8 positions) with 200 µl volume/pipette

Reagents

- Roswell Park Memorial Institute medium (RPMI-1640)
- Fetal Calf Serum (FCS)
- Penicillin 1)
- Streptomycin 1)
- L-glutamine
- 0.05% Trypsin-EDTA
- PBS (Ca and Mg free)
- RPMI-1640 – phenol red free
- MTS – CAS number: 138169-43-4
- $CdSO_4$ (10 mmol/L stock solution provided by Empa)
- Trypan blue*) - CAS number: 72-57-1

1) bought as a ready made mixture PenStr from e.g. Gibco

Reagent Preparation

- Complete cell culture medium:
    - Roswell Park Memorial Institute medium (RPMI-1640) with added:
    - 10% FCS (Fetal Calf Serum)
    - 1 x PenStr ready made mixture with:
        - 10 µg/mL Penicilin
        - 10 µg/mL Streptomycin
    - 0.2 mg/mL L-glutamine.

  This media is termed complete cell culture media for the purpose of this SOP.

  Note: Due to L-glutamine potential degradation, cell culture medium must not be stored over more than 3 weeks.

- $CdSO_4$ Stock Solution: Concentration: 10 mM (stock solution = 100 x working solution) provided by Empa. This stock solution was produced in the following way:
    - 102.64 mg $CdSO_4$ is weighed
    - Transferred in a sterile 50 ml tube
    - Dissolved with 40 ml distilled water
    - --> 40 ml 10mmol/L $CdSO_4$ Store at 4°C (sterile filtration is not necessary)

Cell Culture

All cell cultures are maintained in 75 cm^2 cell culture flasks, in which the cells are passaged at 70-80% confluency every 2-3 days. A549 cells are cultured in complete RPMI-1640 cell culture medium (see Reagent Preparation section) at 37°C, 5% CO_2 in humidified air.

Grow A549-A and A549-B cells (note passage on arrival), which were distributed in frozen form. Upon thawing, cells should be passaged at least 2 times before use in experiments. The cells should not be kept longer than 3 month in culture.

Aliquots of early-passage cells should be frozen down and stored in liquid nitrogen as stock aliquots for later usage.
Note: Immediately after thawing and within the first passage, cell growth may be slow.

A549 cells grow rapidly with a typical population doubling time of 24 h. When the cells are grown in phenol red-containing medium, a change in color from red to yellow could indicate culture overgrowth (i.e. depletion of nutrients). Trypsin is used to remove the cells.

Cell Harvesting

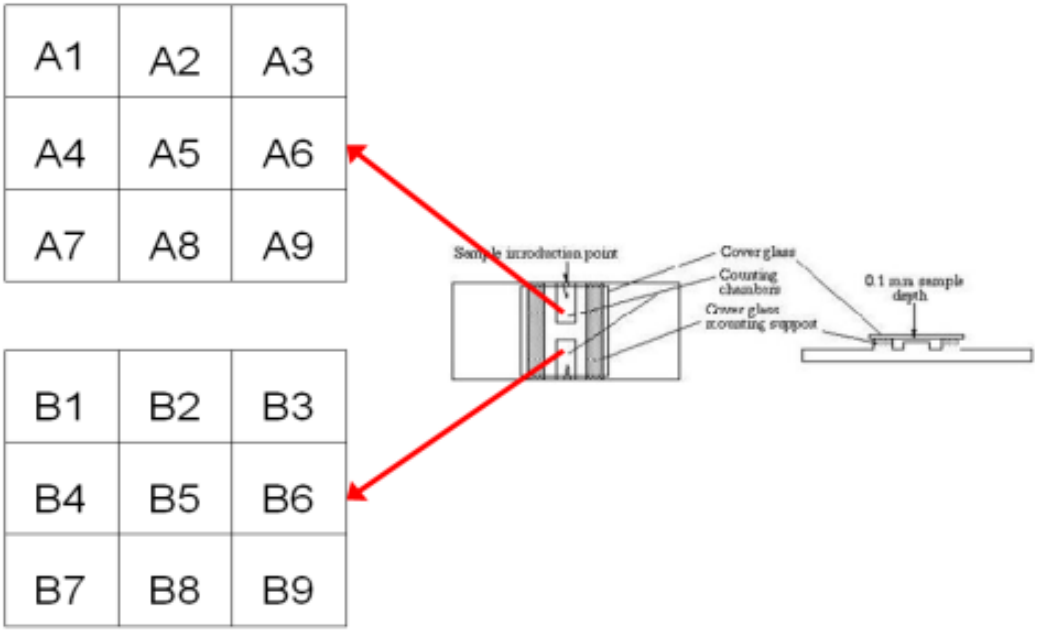
- Cells are grown with 20 mL of complete cell culture media in an incubator under standard conditions (37°C, 5% CO_2 in humidified air).
- Cells are harvest from the cell culture flask by:
    - Wash 2 x the cell culture with 20 mL of PBS
    - Add 2 mL Trypsin-EDTA to the cell culture for 3 minutes
    - Release the cells by vigorous shaking

- Transfer the cells by adding additional 18 mL of the complete cell culture media by washing the bottom of the cell culture flask several times (3 to 5 times)
- Count the cells and monitor doubling rate

Counting Cells

Note: A laboratory can use their established counting method for cells. As long as they have shown that the employed method is equivalently performing than the method outlined afterwards.

1. Harvest cells from cell culture media
2. Sample ~ 20 mL (2 mL Trypsin-EDTA + 18 mL of the complete cell culture media) of cell suspension into 50 mL conical tube using a pipette.
3. Centrifuge cell suspension to precipitate cells as pellet for 5 min at $200\times g = 200\times 9.81\text{N}$.
4. Remove the supernatant and add ~ 1 mL media solution and re-suspend the pellet into the media solution
5. Take 30 µL of cell suspension and add into 30 µL of 0.4 % (w/v) trypan blue/PBS in a 2 mL microcentrifuge tube. Mix the solution by pipetting with 200 µL micropipette.
6. As live cells can also be stained by trypan blue after extended period of time, perform cell counting in 30 minutes.
7. With a cover-slip in place, use a 200 µL micropipette to transfer 10 µL of trypan blue-cell suspension at each end of the hemocytometer. Try to avoid bubbles within the hemocytometer and cover-slip.



Supplemental Protocol S2, Figure 1: *Schematic diagram of a hemocytometer (cell counting chamber)*

8. Count all the cells (non-viable cells stain blue, viable cells will remain opaque) in the four 1mm^2 squares at the corners (A1, A3, A7, A9, B1, B3, B7 and B9 in Fig. 1), which gives total of eight squares for counting. If greater than 25% of cells are non-viable, the culture is not being maintained on the appropriate amount of media. The current cell suspension should be abandoned and cells have to be recultured.
9. If there are less than 50 or more than 200 cells per large square, repeat the procedure adjusting to an appropriate dilution factor.
10. Calculate the number of cells per unit volume (cells/mL) using the following equation.

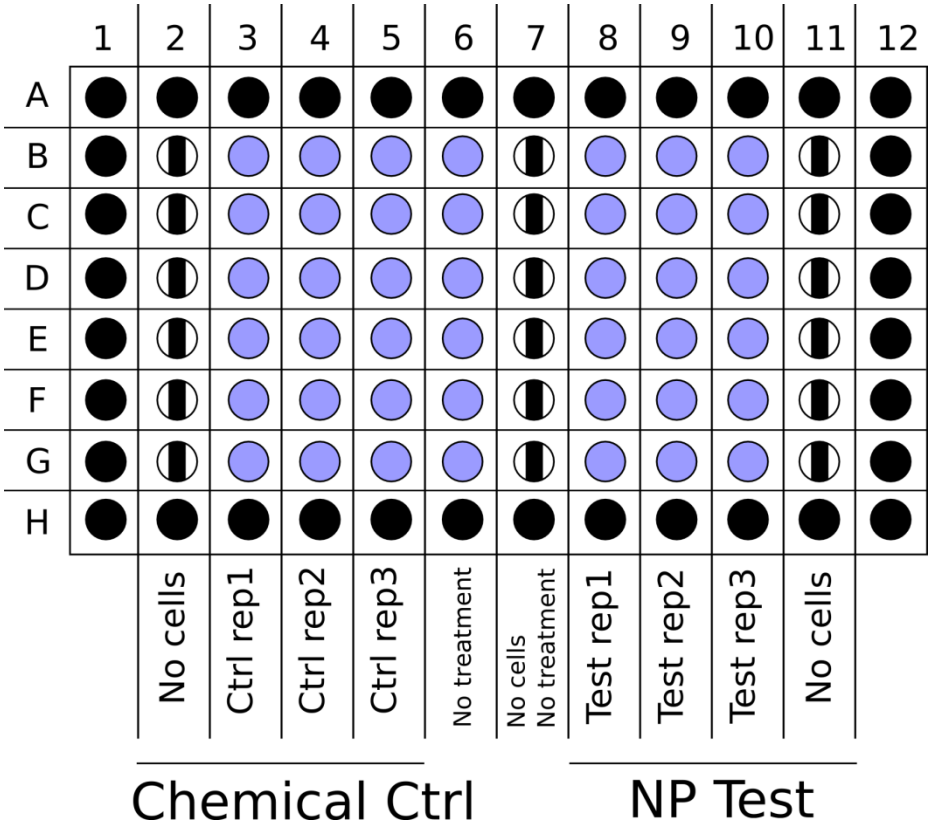
    Number of Cells = Average cell count x the dilution factor x 10^4/mL

11. As an example, we could obtain s/m (standard deviation/average cell count) as 3.0/88.6 = 3.4 % from 6 independent sampling and counting (step 7 & step 8) in recent experiment. (Note: These numbers are not a specification. We might be able to specify them at the end of our interlaboratory comparison)

Procedure

Cell Seeding (24 h)

- 7.5x10^5 cells (7.5x10^4 cells/ml) are suspended in 10 mL of complete cell culture medium for one 96 well plate.
- Cells are seeded at 1.5x10^4 cells/well (200µL) in each of the blue wells (see Figure 2) columns 3 – 6 and 8 – 10). It is important that cells in a single column are seeded with a single multichannel pipetting step.
- Stripped wells contain complete cell culture media only.
- Black wells are whenever possible filled with complete cell culture medium only. If operationally necessary they can be kept empty.



Supplemental Protocol S2, Figure 2: *Seeding of the cells in a 96 well plate – Cells are seeded at 1.5x10^4 cells/well with 200 µL complete cell culture medium in each of the blue wells. Stripped wells contain complete cell culture medium only, whereas black wells could either be filled with complete cell culture medium (better) or stay empty. Cells in a single column should be seeded with a single multichannel pipetting step*

- The 96 well plate with the seeded cells is cultured for 24 h in an humidified incubator at 37° C with 5% CO_2

Preparation of the working Concentrations of the Chemical Control

1. 240 µL sterile H_2O (bi distilled water) are mixed with 23.76 mL complete cell culture medium. This mixture (1) is used in all the following steps for the preparation of the working concentrations of the chemical controls.

2. 40 µL of 10 mmol/L $CdSO_4$ are mixed with 3960 µL of (1) => 100 µmol/L $CdSO_4$
3. 2000 µL of 100 µmol/L $CdSO_4$ are mixed with 2000 µL of (1) => 50 µmol/L $CdSO_4$
4. 2000 µL of 50 µmoll/L $CdSO_4$ are mixed with 2000 µL of (1) => 25 µmol/L $CdSO_4$
5. 2000 µL of 25 µmol/L $CdSO_4$ are mixed with 3000 µL of (1) => 10 µmol/L $CdSO_4$
6. 200 µL of 10 µmol/L $CdSO_4$ are mixed with 1800 µL of (1) => 1 µmol/L $CdSO_4$
7. Mixture (1) is used as 0 µmol/L $CdSO_4$

- 10 mmol/L $CdSO_4$ stock solution has been provided by Empa

- The resulting mixtures are vortexed before used for further dilution steps

Note: The previous prepared working concentrations of the chemical controls are sufficient for the treatment of one 96 well plate. If more than one 96 well plates are treated at the same time, then increase the corresponding amounts with the factor of the number of the treated plates, but keep otherwise the ratios the same.
For example: two 96 well plates:
2x40 µL of 10 mmol/L $CdSO_4$ are mixed with 2x3960 µL of (1) => 100 µmol/L $CdSO_4$:=
80 µL of 10 mmol/L $CdSO_4$ are mixed with 7920 µL of (1) => 100 µmol/L $CdSO_4$)

Preparation of the working Concentrations of the Nanoparticles

1. Complete cell culture medium is directly used without any addition of water. This medium (2) is used in all the following steps for the preparation of the working concentrations of the nanoparticles.

2. 4 µL of 10% PS-NH_2 are mixed with 3996 µL of (2) => 100 µg/mL PS-NH_2
3. 2000 µL of 100 µg/mL PS-NH_2 are mixed with 2000 µL of (2) => 50 µg/mL PS-NH_2
4. 2000 µL of 50 µg/mL PS-NH_2 are mixed with 2000 µL of (2) => 25 µg/mL PS-NH_2
5. 2000 µL of 25 µg/mL PS-NH_2 are mixed with 3000 µL of (2) => 10 µg/mL PS-NH_2
6. 200 µL of 10 µg/mL PS-NH_2 are mixed with 1800 µL of (2) => 1 µg/mL PS-NH_2
7. Medium (2) is used as 0 µg/mL PS-NH_2

- 10% (w/w) PS-NH_2 stock solution has been provided by Empa
- The resulting mixtures are vortexed before used for further dilution steps and again vortexed before they are used for treatment

Note:

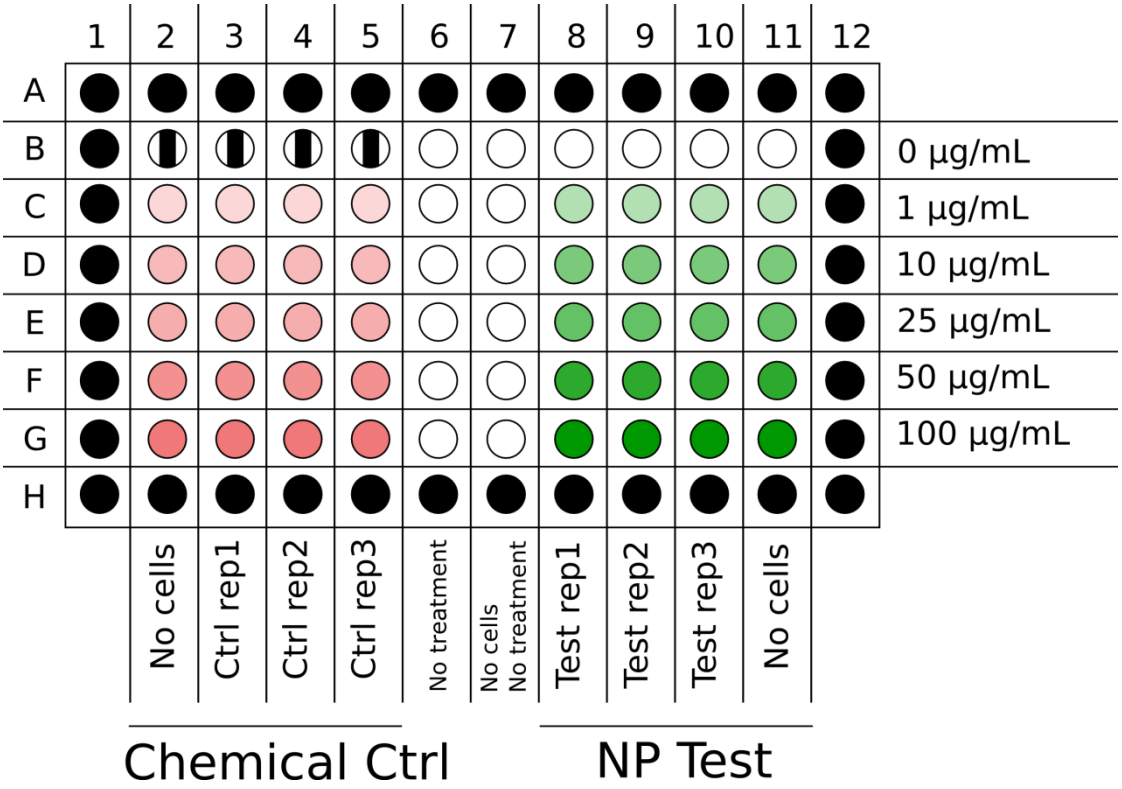
- The previous preparation of working concentrations of the nanoparticle suspensions are sufficient for the treatment of one 96 well plate. If more than one 96 well plates are treated at the same time, then increase the corresponding amounts with the factor of the number of the treated plates, but keep otherwise the ratios the same.
  For example: two 96 well plates:
  2x4 µL of 10% PS-NH_2 are mixed with 2x3996 µL of (2) => 100 µg/mL PS-NH_2 or
  8 µL of 10% PS-NH_2 are mixed with 7992 µL of (2) => 100 µg/mL PS-NH_2
- It is important to record the preparation of the dilution steps in detail. NIST suggests preparing dilutions by putting the tube with the solvent onto a vortexer and then vortexing it while adding the NP containing solution as described in Zook et al (*Nanotoxicology,* 2011, **5**, 517-530).

Treatment of the seeded Cells with NP and Chemical Control

- Working concentrations of the chemical control (see Preparation of the Working Concentration of the Chemical Control section) and of the nanoparticle (see Preparation of the Working Concentration of the Nanoparticles section) have to be ready for direct transfer into a micropipette (200 µL) before the following steps are performed.

- **Attention:** All working concentrations of the nanoparticles have to be vortexed directly before the transfer into the micropipette.

1. Wells of columns 2 – 5 and of columns 8 – 11 are emptied completely by pumping out the supernatant cell culture medium.
2. Columns 8 – 11 are treated with the working concentrations of the nanoparticles according to the plate layout shown in figure 3.
3. Columns 2 – 5 are treated with the working concentration of the chemical control according to the plate layout shown in figure 3.
4. The 96 well plate is culture for 48 h after the treatment in a humidified incubator at 37° C with 5% CO_2.



Supplemental Protocol S2, Figure 3: *Dosing plate layout: white wells contain complete cell culture medium, whereas black wells can either contain complete cell culture medium (better) or stay empty. Stripped wells contain mixture (1). Columns 8 – 11 and 2 – 5 are treated column by column with a 10-channel multichannel pipette (200 µL)*

MTS Cell (viability) Activity Assay

Cellular viability is determined by the MTS assay, which measures the reduction of {3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium} (MTS) in the presence of phenazine methosulfate (PMS). It produces a formazan product that has an absorbance maximum at 490-500 nm. After incubation with the indicated doses of NP for 48 h at 37°C, formazan absorbance is measured at 490 nm.

1. 2.5 mL MTS reagents are mixed with 12.5 mL RPMI-1640 medium (phenol red free). This mixture is called (3). There is no addition of serum, L-glutamine, Penicillin and Streptomycin to the (phenol red free) RPMI-1640 medium.
2. All 96 wells of the plate are emptied completely from the supernatant cell culture medium by pumping.
3. All 96 wells of the plate are filled with 120 µL/well of mixture (3).
4. The 96 well plate is incubated for 60 minutes in an humidified incubator at 37° C with 5% CO_2
5. The absorption measurements are performed with a plate reader at a wavelength of 490 nm.
6. These raw absorption results are sent to Empa for the detailed evaluation of the interlaboratory comparison.

Supplemental Methods

In vitro Sedimentation, Diffusion and Dosimetry Modeling: The main transport processes of particles in suspension are diffusion (Stokes-Einstein Equation) and gravitational settling (Stokes' Law), both of which directly depend on particle size and density. Diffusion is inversely related to particle diameter while sedimentation is driven by particle diameter to the power of two (Teeguarden et al., 2007). Thus, the agglomeration of primary particles is a process that affects particle size, shape and density and therefore directly affects particle transport (Hinderliter et al., 2010; DeLoid et al., 2014). Typically, an agglomerate possesses interparticle pore space, i.e. entrapped media between its constituent primary particles, because the particles are not efficiently packed, which is why they have been modeled as a fractal structure (Sterling et al., 2005; Hinderliter et al., 2010). Interparticle pore space affects both agglomerate porosity and reduces the resulting agglomerate density. The two main sources of interparticle pore space in agglomerates are packing effects and the fractal nature of a particle, the former of which is described by the packing factor (PF) and the latter of which by the fractal dimension (FD), both of which are not well known and not experimentally measurable (Cohen et al., 2012). The packing factor (PF) describes how particles are packed into agglomerates and depends on the monomer shape. The value for the PF is between 0 and 1 (absence of porosity, efficiently packed), and the empiric default is 0.637 for randomly packed spherical monomers. The fractal dimension (FD) depends on how the agglomerate forms through flocculation and takes on values between 1 (rod shaped) and 3 (perfect sphere, porosity = 0), and its empiric default value is 2.3 (Cohen et al., 2012; Hinderliter et al., 2010). The ISDD model based on agglomerate diameter (i.e., using the Sterling equation) was used to estimate the effective NP dose at the plate surface in the presence and absence of serum in the culture media. It uses the agglomerate diameter and FD to calculate agglomerate density, porosity and transport. All of the input parameters used to model the NH_2-PS NPs in the MTS assay are listed in Table S8.

The simulation yields four output values: 1) the fraction of administered dose deposited and the corresponding 2) total number, 3) total surface area (of a sphere) [cm^2] and lastly 4) total mass [μg] in terms of primary NPs deposited. These values were then normalized to the total surface area of the well bottom corresponding to the 96-well plates (0.34 cm^2) to obtain the mass dose [$\mu g/cm^2$], number dose [$\#/cm^2$] and surface area dose [cm^2 NPs/cm^2 well bottom], which were then used to interpret the obtained dose-response relationships and EC_{50} values.

Supplemental Results and Discussion

In vitro Sedimentation, Diffusion and Dosimetry Modeling: ISDD simulations suggest that the delivered dose at the bottom of the exposure vessel is time and treatment dependent (Table S6). The administered NP concentration did not affect the fraction of the delivered dose, which is in line with the underlying model assumptions that do not account for dynamic NP interactions and agglomeration during the simulated exposure period. The fraction of administered dose was highest for smallest particles and decreased with increasing diameters (Table S6), which may be explained by the reduced density of large agglomerates through media entrapment within, approximating that of the media itself, and also suggests that diffusion was the dominant particle transport process. This observation is consistent with previous simulations of carboxylated polystyrene particles of similar sizes (Hinderliter et al., 2010). The fraction of administered dose delivered in the serum-free treatment at 24 h and serum treatments at 48 hours was 0.1846 and 0.0662, respectively (Table S6); uncertainty values calculated based on changing the z-average diameter by 10 % are provided in Table S6. It must be noted, however, that the ISDD model generally overestimates the deposited fraction of administered dose. In the case of carboxylated polystyrene the ratio of simulated to measured rates of transport has been shown to vary from 0.37 up to three-fold, for NP diameters 100 nm to 1100 nm (Hinderliter et al., 2010). This is likely due to the underlying model assumptions, most importantly concerning the bottom boundary condition assuming a 100 % sticky well bottom (i.e. NPs that reach the bottom are immediately internalized by cells and no longer affect particokinetics) and other limitations recently described by DeLoid et al. (2015).
Dosimetrically adjusted EC_{50} values reported in delivered dose metrics are provided in Table S7, and have important implications for the interpretation of the potency of NH_2-PS NPs as well as the biological responses. The adjusted dose metrics are similar between the two exposure conditions suggesting that the differences implied by the conventional administered dose metric are likely due to differential particokinetics in serum and serum-free conditions rather than biological activity of particles/cell sensitivity in serum and serum-free conditions. These findings highlight the importance of integrating the bioavailability of NPs into *in vitro* nanotoxicity testing.

Table S1: Reagent supply

Lab	$CdSO_4$[1)]	NP[2)]	Serum	Medium	Manufacturer for cell culturing containers	96-well plate plastic
EMPA	EMPA	EMPA	Lonza-Brazillian[3)]	Sigma-Aldrich	TPP	TPP
JRC	EMPA	EMPA	Invitrogen – New Zealand	Invitrogen – EU	BD Falcon	BD Falcon
KRISS	EMPA	EMPA	Welgene	Welgene	SPL	SPL
NANOTEC	EMPA	EMPA	Invitrogen-US	Invitrogen-US	Corning	Corning
NIST	EMPA	EMPA	Invitrogen-US	Invitrogen-US	BD Falcon	BD Falcon

1. Hydrated $CdSO_4$ (97%) was dissolved in 18 MΩ water to make a 100 µM solution.
2. NH_2-PS NP were purchased from Bangs as a 10 µg/mL solution in aqueous solution.
3. All serum was heat inactivated except that used by NIST

Table S2: Procedures

Lab	Harvesting	Splitting/Seeding[1)]	Counting Method	Cell resuspension	Washing Step[2)]	Nanoparticle dispersion[3)]	Plate Reader[4)]
EMPA	trypsin	3 day-4 day Seed after 1-2-3-4 days[5)]	Hemocytometer	Rocker	Multiple vacuum aspiration	Vortex/pipette	BioTek
JRC	trypsin	3 day-4 day Seed at split	Hemocytometer	Pipette action	Single pipette aspiration	Vortex/ pipette	FLUOstar Omega (BMG Labtech)
KRISS	trypsin	2 day Seed at split	Hemocytometer	Pipette action	Single pipette aspiration	Vortex/pipette	Molecular Devices
NANOTEC	trypsin	2 day-3 day Seed at split	CASY	Pipette action	Single pipette aspiration	Vortex/pipette	BioTek
NIST	trypsin	3 day-4 day Seed at split	Coulter	Pipette action	Single vacuum aspiration	Vortex/pipette	BioTek

1. Top values are the splitting cycle during routine passaging. The second value is when cells were seeded for experiment round. Cells were split at ≈70% confluence levels and seeded at densities that resulted in 70% confluence after the desired time before the next passage. For example, with a 3 day-4 day passage schedule using T25 flasks, cells were seeded at 200,000 and 110,000 cells/flask for the 3 day and 4 day passage, respectively.
2. Single pipette aspiration was performed with a manual micro pipette. It appears to be gentler than vacuum aspiration as determined by background absorbance measurement in the NP control experiments.
3. Vortex/pipetting refers to the procedure basically described in the protocol and in Zook et al.(Zook et al., 2011)
4. All laboratories tested the repeatability of their plate reader for a single plate and measured a coefficient of variation less than 2%.
5. Approximately the same number of cells that were cultured for 1, 2, 3, or 4 days after splitting were combined and then seeded on the 96 well plate.

Table S3: Cell line characteristics

Cell line	Cell cycle time (h)	Medium volume (μm^3)[1]	Short tandem repeat (STR) analysis[2]
A549-A	22.6±2.2[3]	2327±94	Missing allele 12 (CSF1PO)
A549-B	22.5±2.4[3]	2047±90	In agreement with ATCC

[1]from Coulter counter at passage 7

[2]Promega PowerPlex Fusion STR Kit

[3] These values were averaged over 16 passages for each cell line. These values were not statistically significant ($p < 0.05$), n=3, two-tailed t-test

Table S4: Dynamic light scattering & zeta potential of the NH_2-PS NP

Solvent	DLS (nm)			Zeta potential (mV)		
	0 h	24 h	48 h	0 h	24 h	48 h
De-ionized water[1]	56.6 ±0.9	55.9 ±0.9	54.5 ±0.9	48.8 ±1.9	53.4 ±0.8	48.9 ±1.4
Serum-free media	56.7 ±1.0	61.8 ±0.8	67.5 ±1.3	24.5 ±1.4	23.5 ±1.2	24.1 ±1.7
0.1% FBS media	1100 ±55	2012 ±80	2390 ±91	-0.9 ±0.3	-7.1 ±0.7	-6.8 ±1.1
10% FBS media	1258 ±54	1126 ±37	1258 ±36	-10.7±0.9	-11.2 ±1.6	-11.1 ±1.2

[1]18 MΩ-cm water

Table S5: EC_{50} values for NH_2-PS NP in A549-A and A549-B cells in serum and serum free conditions.

	Mean (mg/L)[1]	Median (mg/L)	95 % Confidence Interval (lower limit, upper limit) (µg/L)
A549-A cells - Serum free			
Consensus	22.5	22.5	16.6, 28.5
Lab A[2]	10.4	10.4	10.1, 11.1
Lab B	21.1	21.1	19.5, 22.6
Lab C	21.2	21.2	20.3, 22.1
Lab D	25.1	25.1	24, 26.4
A549-B cells - Serum free			
Consensus	22.1	22.2	16.9, 27.2
Lab A	22.6	22.6	21, 24.4
Lab B	23.6	23.6	22.2, 24.7
Lab C	23.3	23.3	22, 24.4
Lab D	25.8	25.7	25.1, 27.4
Lab E	15.3	15.4	11.5, 18.8
A549-A cells - Serum			
Consensus	57.7	57.1	47.2, 71.2
Lab A	52.2	52.2	50.7, 54.0
Lab B	52.8	52.7	48.4, 58.1
Lab C	61.6	61.0	54.5, 72.8
Lab D	64.1	63.3	53.9, 79.6
A549-B cells - Serum			
Consensus	52.6	52.6	44.1, 62.6
Lab A	47.6	47.5	46.2, 48.7
Lab B	49.7	49.6	46.7, 52.8
Lab C	62.7	62.4	58.9, 68.0
Lab D	56.1	56.0	52.1, 61.1
Lab E	47.2	47.3	45.2, 49.2

[1]The mean, median, and 95 % confidence intervals for the EC_{50} values were calculated by fitting all rounds from each from laboratory with a Bayesian statistical model. The asymmetric uncertainty is shown for the median value. The consensus values were generated by using all the interlaboratory data in a Bayesian statistical model

[2] outlier not included in the determination of the consensus value

Table S6: Delivered doses at 24 h and 48 h in the serum-free and serum treatments, respectively, expressed in terms of the fraction delivered, and the number, surface area (of a sphere) and mass dose per cm² well bottom in terms of primary NPs for an administered dose of c_{adm} = 100 µg/mL.

	fraction delivered	number dose [#/cm²]	surface area dose [cm² NPs /cm²][1]	mass dose [µg/cm²]
RPMI - 24 h, d_H = 56 nm	0.1942	1.18E+11	11.65	11.42
RPMI - 24 h, d_H = 62 nm	0.1846	1.12E+11	11.08	10.86
RPMI - 24 h, d_H = 68 nm	0.1764	1.07E+11	10.59	10.37
RPMI/10% FBS - 48 h, d_H = 1094 nm	0.0686	4.18E+10	4.12	4.04
RPMI/10% FBS - 48 h, d_H = 1214 nm	0.0662	4.04E+10	3.98	3.90
RPMI/10% FBS - 48 h, d_H = 1334 nm	0.0643	3.91E+10	3.86	3.78

1 The surface area dose is defined as the surface area of a sphere per cm^2 well bottom, in terms of primary NP size. To obtain the number of deposited monolayers, this value can be divided by 4 (assuming full packing, 100 % surface coverage) or 8 (assuming random close packing, 50 % coverage).

Table S7: Dosimetrically corrected mean EC_{50} values for d_H = 62 nm (RPMI) and d_H = 1214 nm (RPMI/10 % FBS).

	mean EC_{50} [µg/mL]	corrected EC_{50} number dose [#/cm²]		corrected EC_{50} SA dose [cm² NPs /cm²]		corrected EC_{50} mass dose [µg/cm²]	
		24 h	48 h	24 h	48 h	24 h	48 h
A549-A cells, serum- free							
Consensus	**22.5**	**2.53E+10**	**-**	**2.49**	**-**	**2.44**	**-**
Lab A	10.4	1.17E+10	-	1.15	-	1.13	-
Lab B	21.1	2.37E+10	-	2.34	-	2.29	-
Lab C	21.2	2.38E+10	-	2.35	-	2.30	-
Lab D	25.1	2.82E+10	-	2.78	-	2.73	-
A549-B cells, serum- free							
Consensus	**22.1**	**2.49E+10**	**-**	**2.45**	**-**	**2.40**	**-**
Lab A	22.6	2.54E+10	-	2.50	-	2.45	-
Lab B	23.6	2.65E+10	-	2.62	-	2.56	-
Lab C	23.3	2.62E+10	-	2.58	-	2.53	-
Lab D	25.8	2.90E+10	-	2.86	-	2.80	-
Lab E	15.3	1.72E+10	**-**	1.70	**-**	1.66	**-**
A549-A cells, serum							
Consensus	**57.7**	**-**	**2.33E+10**	**-**	**2.29**	**-**	**2.25**
Lab A	52.2	-	2.11E+10	-	2.08	-	2.03
Lab B	52.8	-	2.13E+10	-	2.10	-	2.06
Lab C	61.6	-	2.49E+10	-	2.45	-	2.40
Lab D	64.1	-	2.59E+10	-	2.55	-	2.50
A549-B cells, serum							
Consensus	**52.6**	**-**	**2.12E+10**	**-**	**2.09**	**-**	**2.05**
Lab A	47.6	-	1.92E+10	-	1.89	-	1.85
Lab B	49.7	-	2.01E+10	-	1.98	-	1.94
Lab C	62.7	-	2.53E+10	-	2.49	-	2.44
Lab D	56.1	-	2.26E+10	-	2.23	-	2.19
Lab E	47.2	**-**	1.90E+10	**-**	1.88	**-**	1.84

Variations in d_H (± 10 %) introduce variabilities of + 5.17 % (d_H = 56 nm) and – 4.47 % (d_H = 68 nm) in serum-free treatments (RPMI) as well as + 3.61 % (d_H = 1094 nm) and – 3.00 % (d_H = 1334 nm) in serum treatments (RPMI/10 % FBS). The difference in variability is not equal because size and particle transport do not correlate linearly.

Table S8: ISDD model input parameters for the MTS assay in serum and serum-free treatments. Values for media viscosity and density are taken from literature (DeLoid et al., 2014; Hinderliter et al., 2010).

	RPMI	RPMI/10 % FBS
1) primary NP properties		
1.a) diameter [nm]	56	56
1.b) density [g/mL]	1.05	1.05
2) NP agglomerate properties		
2.a) diameter [nm]	62 ± 6	1214 ± 120
2.b) density [g/mL]	NA	0.03022
2.c) fractal dimension	2.3	2.3
2.d) packing factor	0.637	0.637
3) exposure medium properties		
3.a) dish depth [m]	0.00588	0.00588
3.b) volume [mL]	0.2	0.2
3.c) temperature [K]	310	310
3.d) viscosity [Ns/m^2]	0.00069	0.00074
3.e) density [g/mL]	1.0072	1.0084
3.f) exposure time [h]	24 h	48 h

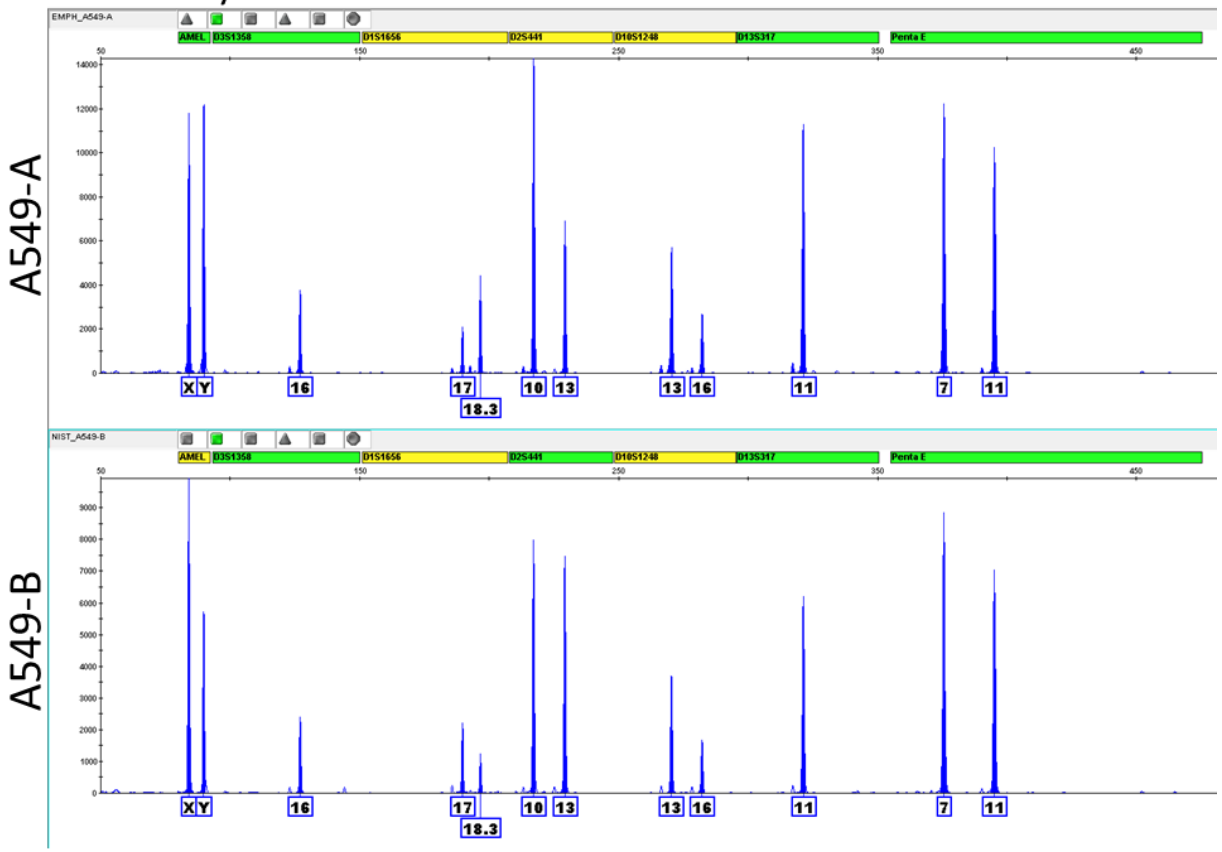
Table S9: EC_{50} values for $CdSO_4$ in A549-A and A549-B cells in serum and serum free conditions.

	Mean (µmol/L)[1]	Median (µmol/L)	95 % Confidence Interval (lower limit, upper limit) (µmol/L)
A549-A cells - Serum free			
Consensus	24.9	24.9	22.7, 27.0
Lab A [2]	24.4	24.5	23.1, 25.0
Lab B	24.9	25.0	23.9, 26.0
Lab C	24.0	24.2	21.7, 25.0
Lab D	26.2	25.9	25.1, 29.2
A549-B cells - Serum free			
Consensus	49.7	49.9	47.5, 51.5
Lab A	25.6	25.5	25.0, 26.9
Lab B	50.0	49.9	48.0, 51.2
Lab C	50.1	50.0	48.9, 51.7
Lab D	50.0	49.9	48.5, 51.2
Lab E	49.3	49.9	43.9, 50.9
A549-A cells - Serum			
Consensus	58.8	56.2	50.5, 82.5
Lab A	59.7	56.2	50.6, 88.5
Lab B	55.6	55.2	50.6, 65.2
Lab C	59.6	56.2	50.6, 88.8
Lab D	59.7	56.0	50.6, 91.0
A549-B cells - Serum			
Consensus	77.0	77.2	54.3, 99.4
Lab A	76.0	74.4	53.8, 99.3
Lab B	76.0	74.2	53.7, 99.3
Lab C	76.8	75.2	53.8, 100.2
Lab D	75.7	73.6	53.3, 99.3
Lab E	76.1	74.5	53.8, 99.3

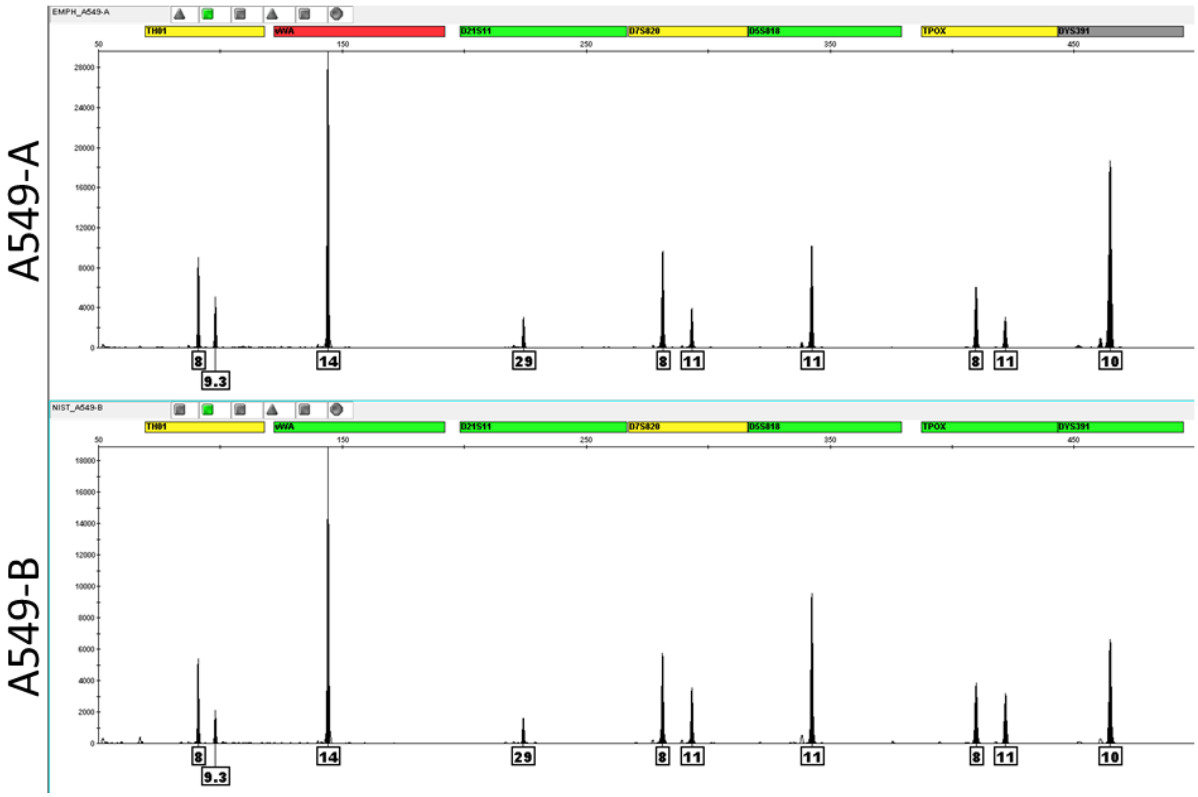
[1]The mean, median, and 95 % confidence intervals for the EC_{50} values were calculated by fitting all rounds from each from laboratory with a Bayesian statistical model. The asymmetric uncertainty is shown for the median value. The consensus values were generated by using all the interlaboratory data in a Bayesian statistical model

[2] outlier not included in the determination of the consensus value

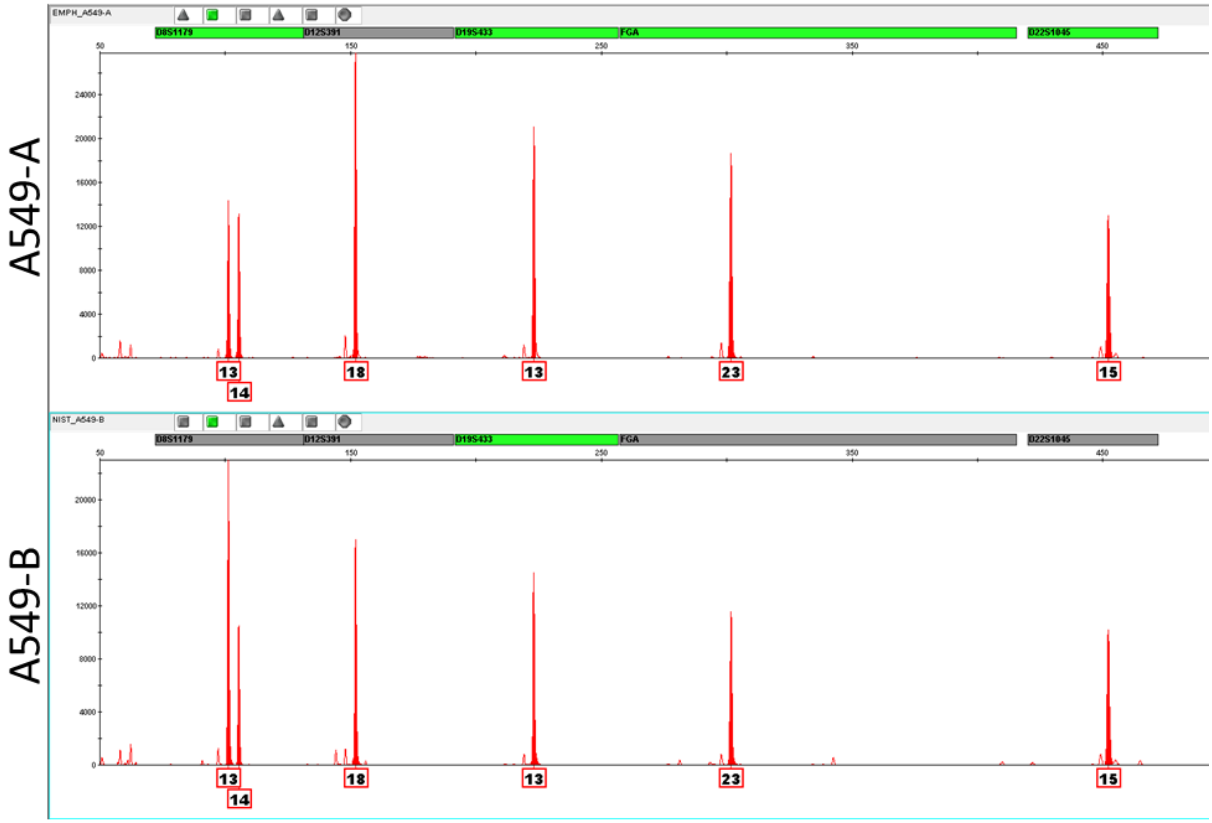
a. FAM dye



b. NED dye



c. PET dye



d. VIC dye

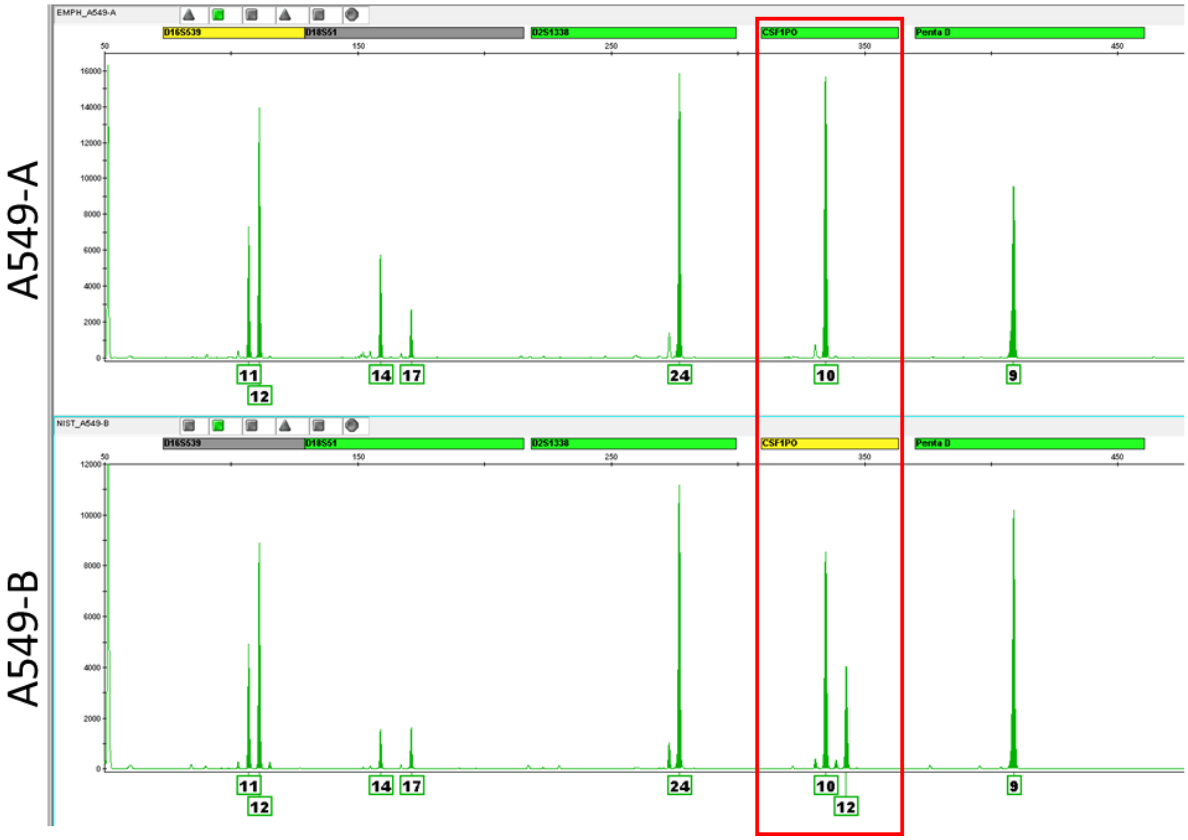


Figure S1: A549 Cell Line Authentication by Short Tandem Repeat DNA sequences for cell lines A549-A and A549-B. Both cell lines were authenticated and compared by their STR genotypes (PowerPlex Fusion, Promega, Madison, WI). The results indicate that the A549-B cells have the expected STR markers as described by the vendor (ATCC, Manassas, VA). Interestingly, the A549-A cells have 23 identical STR markers with the exception of a single drop out of the 12 allele at the CSF1PO locus (VIC Dye Channel, Figure S3d). This locus is located on chromosome 5 and suggests that A549-A cell line may have a mutation in the primer binding site, which may have caused failure in the amplification of the 12 allele.

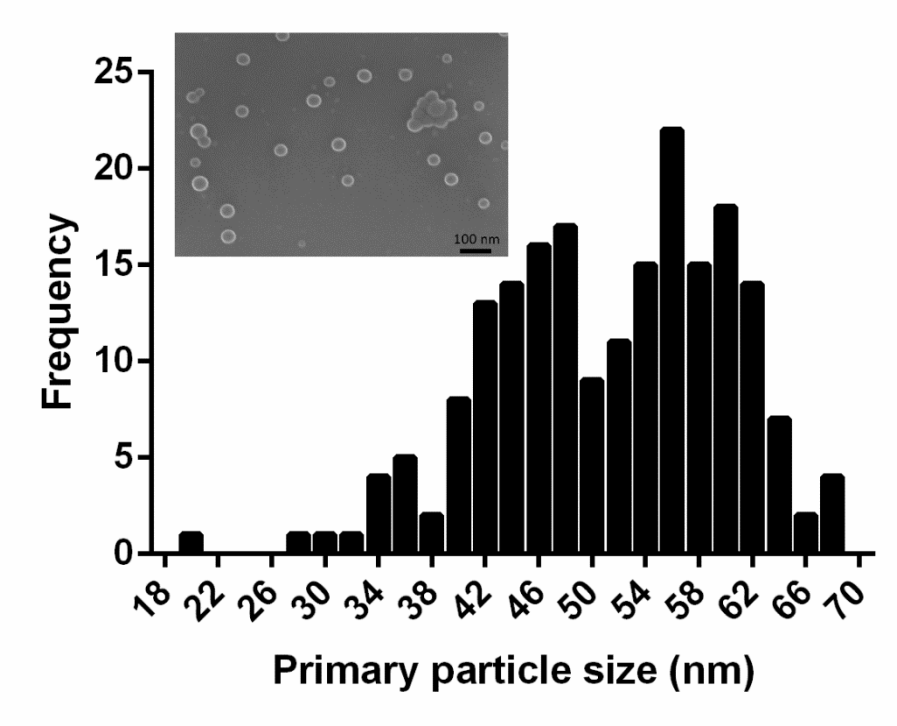


Figure S2: Scanning electron micrograph and primary particle size histogram for the NH_2-PS NP. Reprinted by permission of The Royal Society of Chemistry (Hanna et al., 2016).

References

Cohen, J., DeLoid, G., Pyrgiotakis, G. et al. (2012). Interactions of engineered nanomaterials in physiological media and implications for in vitro dosimetry. *Nanotoxicology 7*, 417-431.

DeLoid, G., Cohen, J. M., Darrah, T. et al. (2014). Estimating the effective density of engineered nanomaterials for in vitro dosimetry. *Nature Communications 5*, 10. http://dx.doi.org/10.1038/ncomms4514

DeLoid, G. M., Cohen, J. M., Pyrgiotakis, G. et al. (2015). Advanced computational modeling for in vitro nanomaterial dosimetry. *Particle and Fibre Toxicology 12*, 1.

Hanna, S. K., Cooksey, G. A., Dong, S. et al. (2016). Feasibility of using a standardized *Caenorhabditis elegans* toxicity test to assess nanomaterial toxicity. *Environ Sci: Nano* in press.

Hinderliter, P., Minard, K., Orr, G. et al. (2010). ISDD: A computational model of particle sedimentation, diffusion and target cell dosimetry for in vitro toxicity studies. *Particle and Fibre Toxicology 7*, 36.

Sterling, M. C., Bonner, J. S., Ernest, A. N. et al. (2005). Application of fractal flocculation and vertical transport model to aquatic sol–sediment systems. *Water research 39*, 1818-1830.

Teeguarden, J. G., Hinderliter, P. M., Orr, G. et al. (2007). Particokinetics in vitro: Dosimetry considerations for in vitro nanoparticle toxicity assessments. *Toxicological Sciences 95*, 300-312. http://dx.doi.org/10.1093/toxsci/kfl165

Zook, J. M., MacCuspie, R. I., Locascio, L. E. et al. (2011). Stable nanoparticle aggregates/agglomerates of different sizes and the effect of their size on hemolytic cytotoxicity. *Nanotoxicology 5*, 517-530. http://dx.doi.org/10.3109/17435390.2010.536615