

Protocols for testing of metal oxide NP in mammalian tissue culture cells to study the Cell viability based on an oxidative stress paradigm

C. Biological assays on RAW 264.7 (murine macrophage cell line):

C.1. Cell culture and co-incubation with NP

All cell cultures are maintained in 25 cm² cell culture flasks, in which the cells were passaged at 70-80% confluency every 2 days. RAW 264.7 cells (ATCC # TIB-71) are cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Cat. # 11995-065) containing 10% (10:1 mixture) FCS (Fetal Calf Serum) (Gemini Cat.# 100-106), 100 U/ml penicillin (Invitrogen Cat.# 10378-016), 100 µg/ml streptomycin (Invitrogen Cat.# 10378-016), and 2 mM L-glutamine (Invitrogen Cat.# 10378-016). BEAS-2B (ATCC # CRL-9609) cells are cultured in BEGM (Lonza Cat. # CC-3171) in type I rat tail collagen-coated flasks or plates.

All the NP solutions are prepared fresh from stock solutions (10 mg/ml). The stock solutions are made up from dry powder unless already in solution. Depending on the volume concentration of particles in solution, an appropriate volume of WFI grade H₂O is added to the appropriate volume of particles. Dry powder NP are weighed using a chemical scale (Mettler Toledo Cat.# AL54) that can weigh out to 100 µg of material and brought up at a concentration equal to 10 mg/ml using cell WFI grade H₂O (Irvine Scientific, Cat.# 9309). For cell culture experiments this stock is vortexed (or sonicated using Sonicating Water Bath) immediately before diluting into complete cell culture medium (that contains 10% fetal calf serum). For instance, a 50 µg/ml exposure dose is made by adding 5 µl of the NP into 1 ml of complete culture medium. The NP suspension is then sonicated for 10 seconds prior to cellular exposure assays.

Proposal: For subculture, for RAW 264.7 cells, pipetting is enough because they are loosely attached; for BEAS-2B cells, collect cell using trypsin/EDTA and pipetting. No scraping is needed.

C.2. MTS cell viability assay

Cellular viability is determined by the MTS assay, which looks at the reduction of {3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium} (MTS) to formazan in viable cells¹. After incubation with the indicated doses of NP for various lengths of time at 37°C, formazan absorbance is measured at 490 nm. While a brief overview of the protocol is described here, the manufacturer's protocol is available online at <http://www.promega.com/tbs/tb245/tb245.pdf>

1. 25×10^3 cells are plated onto 96 multi-well plates (Falcon Cat.# 353072) and grown for 24 hours until 70% confluent. NP are co-incubated with the cells for 6 and 16 or 24 hrs (so, two time points) at concentrations of 1, 10, 25, 50, and 100 µg/ml. Controls are untreated cells that are harvested at both time points.

2. Thaw the CellTiter 96® Aqueous One Solution Reagent from an aliquoted source immediately prior to use. Remove the original cell culture supernatant (containing the NP) from

the wells and replace with 100 μ l of fresh complete DMEM that is phenol red free (Gibco/Invitrogen, Cat. # 21063-029). Add 20 μ l of the MTS reagent directly to each well.

3. The 96-well plates are incubated for 30 to 60 min at 37°C in a humidified, 5% CO₂ atmosphere.

4. Formazan absorbance is measured at 490 nm using a microtiter plate reader (Bio-Tek, Winooski, VT). The amount of colored product formed is proportional to the number of live cells in culture.

5. An additional control, namely the MTS reagent mixed with NPs only, should be performed at some point to ensure that there is no interference with the reagent.

6. Data analysis: Data are collected using software from KC Junior™. Data are expressed as % cell viability. Mean absorbance of non-exposed cells (control cells) serves as the reference value for calculating 100% cellular viability. If x = mean value for control cells then the % cellular viability for samples (s) is calculated using the following equation: $s = (\text{mean value of sample}/x) \times 100$.

C.4. Cellular staining with fluorescent probes and the performance of flow cytometry to assess a series of biological responses that are triggered by NP-induced cellular injury.

For flow cytometry experiments, 2.5×10^5 cells are plated on 24-well plates (Falcon Cat.# 353078) for the indicated time periods for each of the tests below. RAW 264.7 cells are removed by gentle pipetting. The list of dyes below is used according to manufacturer's protocols. Flow cytometry is performed using a FACScan or LSR (Becton Dickinson, Mountain View, CA) equipped with a single 488 nm argon laser.

C.4.1. CM-H₂DCFDA (assessment of cellular H₂O₂ production)

(Molecular Probes Cat. # C6827)

While a brief overview of the protocol is described here, the manufacturer's protocol is available online at <http://probes.invitrogen.com/media/pis/mp36103.pdf>. The reagent, CM-H₂DCFDA, is referred to below as DCF.

Note: Do not dissolve products until immediately prior to use.

1. 2.5×10^5 cells are plated on 24-well plates (Falcon Cat.# 353078) and grown for 24 hours to 70% confluency in phenol red-free medium prior to exposing to NP at various concentrations for the indicated time periods (1, 3, 6, and 16 or 24 hrs) at concentrations of 1, 10, 25, 50, and 100 μ g/ml.
2. Cells without particles (for each time point) are used as negative controls. Cells treated with 0.5 mM tert-butyl hydroperoxide (t-BuOOH) are used as the positive control.
3. Shortly before performing the flow-cytometry analysis, reconstitute the ROS indicator (DCF) in DMSO to make a concentrated stock solution. Keep tightly sealed until ready to use. This is then diluted to 2.5 μ M DCF in DMEM/10%FBS.
4. Following incubation with NP at the desired concentration and for the required length of time, the original cell culture supernatant (containing the NP) is removed from the wells and replaced with 500 μ l of the prepared 2.5 μ M DCF in DMEM/10%FBS. Cells are incubated with the reagent at 37 °C in the dark for 30 min.
5. Remove the DCF; return the cells to prewarmed growth medium and place in an incubator until they are analyzed. Analysis should occur as soon as possible after loading

the cells with DCF and the study planned in such a way that no more than 20-30 samples are analyzed at any one time.

6. After gentle pipetting to remove cells from the culture wells, they are analyzed on a flow cytometer under channel FL-1 (collect 10,000 events), along with the appropriate negative and positive controls (see above or manufacturer's suggestions). An additional control, namely the DCF reagent mixed with NPs only, should be performed at some point to ensure that there is no interference with the reagent.
7. Data are expressed as fold increase in Mean Fluorescence Intensity (MFI) from control (no NP).

Approximate Fluorescence Excitation and Emission, in nm: 492–495/517–527.

C.4.8. Propidium Iodide (assessment of cell death)

(Molecular Probes Cat.# P3566)

While a brief overview of the protocol is described here, the manufacturer's protocol is available online at <http://probes.invitrogen.com/media/pis/mp01304.pdf>

1. 2.5×10^5 cells were plated on 24-well plates (Falcon Cat.# 353078) and grown for 24 hours to 70% confluency prior to exposing to NP at various concentrations (1, 10, 25, 50, and 100 $\mu\text{g/ml}$) for the indicated time periods (6 and 16 or 24 hrs).
2. Cells without treatment are used as the negative control. Cells treated with 0.5 mM tert-butyl hydroperoxide (t-BuOOH) are used as the positive control.
3. To make a stock solution from the solid form, dissolve propidium iodide (PI; MW = 668.4) in cell WFI grade H_2O (Irvine Scientific, Cat.# 9309) at 1 mg/mL (1.5 mM) and store at 4°C , protected from light. When stored properly, solutions are stable for at least six months. Immediately prior to the experiment, the stock is diluted to 47.5 $\mu\text{g/ml}$ propidium iodide (PI) in DMEM/10%FBS.
4. Following incubation with NP at the desired concentration and for the required length of time, the original cell culture supernatant (containing the NP) is removed from the wells, and replaced with 500 μl of 47.5 $\mu\text{g/ml}$ PI in DMEM/10%FBS.
5. Samples should be immediately analysed on a flow cytometer under the red channel, not later than 2 hours from the addition of PI. Cells are removed from the cell culture wells with gentle pipetting.
6. The percentage of cells at different PI content is compared to that of untreated cells. A control with only the particles should be performed to ensure that PI will not bind to particle surface, thus affecting the obtained values.

Approximate fluorescence excitation/emission maxima: 535/617 nm, bound to nucleic acids

Comment [A1]: Anna: I normally store it at -20°C

References

1. Xia, T.; Kovochich, M.; Liong, M.; Zink, J. I.; Nel, A. E. Cationic Polystyrene Nanosphere Toxicity Depends on Cell-Specific Endocytic and Mitochondrial Injury Pathways. *ACS Nano* **2008**, 2 (1), 85-96.