Combining Cytotoxicity Assessment and *Xenopus laevis* Phenotypic Abnormality Assay as a Predictor of Nanomaterial Safety

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SIGNIFICANCE STATEMENT: Nanoparticles are being produced for an ever-increasing range of applications and with such growth comes a need to efficiently assess any potential toxicity associated with these new materials. Here we describe in detail a step-by-step protocol that can be used to rapidly and effectively assess nanotoxicity, by combining mammalian cytotoxicity assessment with vertebrate abnormality scoring using *X. laevis* embryos. We have previously demonstrated that this approach is effective at determining low-toxicity nanomaterials in mice (Webster et al., 2016). This protocol can be used as a rapid screening approach for newly developed nanomaterials, with high predictive power for determining nanoparticle safety in vertebrate systems.

ABSTRACT

The African clawed frog, *Xenopus laevis*, has been used as an efficient pre-clinical screening tool to predict drug safety during the early stages of the drug discovery process. *X. laevis* is a relatively inexpensive model that can be used in whole organism high-throughput assays whilst maintaining a high degree of homology to the higher vertebrate models often used in scientific research. Despite an ever-increasing volume of biomedical nanoparticles (NPs) in development, their unique physico-chemical properties challenge the use of standard toxicology assays. Here, we present a protocol that directly compares the sensitivity of *X. laevis* development as a tool to assess potential NP toxicity by observation of embryo phenotypic abnormalities/lethality after NP exposure to *in vitro* cytotoxicity obtained using mammalian cell lines. In combination with conventional cytotoxicity assays, the *X. laevis* phenotypic assay provides accurate data to efficiently assess the safety of a novel biomedical NP.

Keywords: Nanoparticles • nanotoxicity • physicochemical characterisation of nanoparticles • cytotoxicity • *Xenopus laevis* embryos

INTRODUCTION

The research and application of biomedical NPs is a rapidly evolving discipline (De Jong and Borm, 2008). For many, it is believed that biomedical nanomaterials can act as advantageous

tools in the treatment of several disease states. In particular, the unique physico-chemical properties of NPs makes them an ideal therapeutic and diagnostic tool in oncology by overcoming the limitations of conventional therapies, as we have previously discussed (Bombelli et al., 2014). The main advantages of using biomedical NPs as drug delivery systems include targeted drug delivery, increased biocompatibility and a decrease in drug toxicity, whilst maintaining or improving the therapeutic effect. However, as a result of the high surface area-to-ratio volume and complex composition of the nanomaterial, NPs can be highly reactive, where combinations of NP size, shape, material, and functionalisation, can result in toxicity within a biological systems (Lewinski et al., 2008; Nystrom and Fadeel, 2012).

Conflicting information regarding NP safety for a given material can impede the progression of a NP from the early stages of formulation development through to the clinic. Inconsistencies in NP toxicity data are largely attributable to a lack of a standardised protocol for nanotoxicity assessment. Firstly, full characterisation of a NP system (including size, surface charge, and stability in assay buffers) is required to understand the fate of the NP in a biological system and its potential to cause toxicity. Different early developmental models, such as *Xenopus* species (Bacchetta et al., 2014; Hu et al., 2016; Mouchet et al., 2008; Tussellino et al., 2015; Webster et al., 2016) and zebrafish (George et al., 2011; Liu et al., 2012; Rizzo et al., 2013), have been explored as systems that can provide rapid, accurate, cost effective and abundant data for NP toxicology assessment. *X. laevis* (the African clawed frog) is a species that produce large quantities of embryos allowing them to be used in a high-throughput style assay to gain toxicology data relatively quickly. Furthermore, with an individual embryo size at early developmental stages of ~1 mm, they are well suited for use in a multi-well format. *X. laevis* has the advantage of being evolutionary closer to humans

than other early models such as *Caenorhabditis elegans*, *Drosophila*, and zebrafish (Wheeler and Brandli, 2009). Although mouse models, as the gold standard, are evolutionary closer to humans than *X. laevis*, they are expensive and not a viable option to test numerous NPs over a wide range of concentrations, as far fewer embryos are produced compared to *X. laevis*.

Here we provide a detailed protocol for the use of *X. laevis* embryos in conjunction with cytotoxicity analysis, for highlighting potential NP toxicity by observing phenotypic abnormalities/lethality in response to NP exposure. *X. laevis* development is well documented (Nieuwkoop and Faber, 1967), making it easy to detect when toxicity-induced deviation from normal embryo development has occurred. The rationale for this approach has previously been described (Webster et al., 2016) and involves a combined assessment of cytotoxicity with *X. laevis* abnormality assessment in response to NP treatment, which offers a sensitive nanotoxicity model to bridge standard *in vitro* assessment alone with further rodent testing (Fig. i). Specifically, this methodology incorporates physicochemical characterization of nanomaterials, followed by rapid cytotoxicity and phenotypic abnormality assessment as an indicator of nanotoxicity prior to later testing in mammalian systems.

BASIC PROTOCOL 1 – PHYSICOCHEMICAL CHARACTERISATION OF NPs

This protocol describes the necessary steps to prepare nanoparticle (NP) dispersions suitable for toxicological characterization by cytotoxicity and *X. laevis* phenotypic scoring assays. This protocol is designed to be adaptable to different types of nanoformulations (thus it is not addressed to a specific typology of NPs), but is to be used for NPs dispersed in aqueous solutions. Physical-chemical characterization of NP dispersions is a critical step in a nano-safety assessment protocol (Azhdarzadeh et al., 2015), in particular the experiments need to be performed not only in the NP dispersion medium, but also in the fluids in which the NPs

will be dispersed during the biological assays. It is also important to monitor the colloidal stability of the NP dispersions over the duration of the nanotoxicity assessment period to detect any potential agglomeration effects over time (Cho et al., 2013). Generally, NP dispersions are commonly characterized in terms of hydrodynamic size of the particles through Dynamic Light Scattering (DLS) measurements. To better interpret DLS results it is also necessary to perform Transmission Electron Microscopy (TEM) on the dried samples for evaluating the morphology and size of a single NP. The presence of biomolecules (i.e. proteins) in the biological fluids affects the DLS results by producing a background signal, thus such experiments should be performed at a maximum protein concentration used in the nanotoxicity experiments (i.e. 10% v/v serum used in GM), but not in pure serum as in that case the protein signal overcomes that deriving from the NPs. Moreover, it has been shown that the presence of proteins or other biomolecules in the biological fluids affects the physical-chemical properties of the NPs through the formation of a protein corona around the NPs (Cedervall et al., 2007; Monopoli et al., 2012). Thus, the analysis of DLS data in biological fluids can be more complex than in physiological buffer solutions. In fact, even if DLS is a good technique for testing the stability of NP dispersions in biological fluids, it does not give a quantitative estimation of the size of such complexes (as it cannot distinguish among dimer, trimer or agglomerates of protein-NP complexes). For this purpose it would be necessary to implement the NP characterization with different analysis such as Differential Centrifugal Sedimentation (Walczyk et al., 2010) or Fluorescence Correlation Spectroscopy (Rocker et al., 2009), which is beyond the interest of this protocol.

Materials

NP stock dispersions (concentrations and nanomaterials tested are to be pre-determined

by the experimenter)

Disposable DLS cuvettes

Dynamic Light Scattering apparatus

PBS (see recipe)

Mammalian cell culture growth media (GM; see basic protocol 2 for further details)

0.1X Marc's Modified Ringer's (MMR; see recipe)

1. Prepare the DLS cuvettes cleaning them with autoclaved Milli-Q-purified H_20 (d.H_20) and then dry with particular care to protect them from dust.

2. Transfer the NP dispersions to the DLS cuvettes (necessary volume depends on the DLS apparatus) and dilute them if it is necessary. The solvent used to dilute the NP dispersions must be dust free as much as possible.

IMPORTANT NOTE: Never touch the middle-bottom part of the cuvettes with hands, but always manage them touching them in their upper edge.

The choice of the optimal concentration for DLS measurements should be based on both experimental and technical considerations. A concentration as much as possible similar to those used in the biological assays should be chosen (usually the most concentrated dose used in vitro *is the safest choice to detect possible NP agglomeration).*

It is recommended to run a quick test for evaluating the averaged scattered intensity of the chosen dilution that should be above 20 kcounts/s for be statically significant. If it is lower than that value, a more concentrated sample should be prepared.

3. Set the temperature to the desired value according to that at which the biological experiments are performed.

In this context these temperatures will be 37°C for mammalian and 12-23°C for X.

laevis work.

4. The NP dispersion in the cuvette should be left to rest in the sample holder for approximately 10 minutes before the measurement to reach the desired temperature and allow the eventual dust to sediment.

5. Measure the scattered intensity at a set angle of detection. Generally, the most used apparatus can measure the scattered intensity at a fixed angle (either 90° or 173°), but there are also more advanced instruments that permit multi-angle detection, in that case it is better to measure the scattered intensity at different angles (Fig ii). The detected signal will be automatically sent to the correlator, which produces the auto-correlation function of the scattered intensity $g_2(q,t)$ for each angle (equation 1):

$$g_2(q,t) = \frac{\langle I^*(q,0) I(q,t) \rangle}{\langle I(q,0)^2 \rangle} \quad (1)$$

where:

$$q = \frac{4\pi n}{\lambda} \sin(\theta/2)$$

…is the scattering vector (with θ the detection angle, λ the wavelength of the incident light and n the solvent refractive index).

2 Analyze the auto-correlation functions to extract the NP hydrodynamic size by available analysis softwares. The analysis of the auto-correlation functions at each angle gives a decay rate $\Gamma(s^{-1})$ related to the NP dynamics and related to the translational diffusion coefficient, D, through the following equation for Brownian systems (equation 2):

$$\Gamma(s^{-1}) = D \cdot q^2 \quad (2)$$

Thus reporting the decay rates versus the scattering vectors the slope of the obtained curve is the translation diffusion coefficient. The NP hydrodynamic radius, r_H, can be determined through the Stokes-Einstein relationship (equation 3):

$$D=k_BT/6\pi\rho r_H \quad (3)$$

Where T is the experimental temperature and ρ the viscosity of the solvent.

IMPORTANT NOTE: the fitting analysis of the auto-correlation functions for determining the decay rates must be carefully chosen. If the auto-correlation function is monomodal (the sample is mostly composed of a single population of NPs of the same size), a Cumulant method (Koppel, 1972) can be used. This fitting analysis gives an averaged $<\Gamma>$ together with a polydispersity index (PDI). If the PDI is <0.2-0.25, it is reasonable to use this method. If the PDI is >0.25 the sample is either very polydisperse or composed of two or more populations and an alternative method must be used. The most common is the algorithm CONTIN (Provencher, 1982)-based on the Laplace transform of the auto-correlation function. This method gives a size-distribution of the NP dispersion distinguishing different particle populations differing in scattered intensities of at least 1:10^{-5}. For monomodal polydisperse samples the two methods should give comparable results.

SUPPORT PROTOCOL 1 – TEM FOR NP CHARACTERISATION

As highlighted in Basic Protocol 1, a TEM study should be done on the NP stock dispersion for evaluating NP morphology and better interpreting DLS results. TEM analysis allows the determination of the size of single NPs that can be used for understanding the NP size

distribution obtained by DLS and highlight possible agglomeration effects. TEM equipment comprises of complex instrumentation and usually a dedicated person(s) is/are responsible for its maintenance and running experiments in a core facility within institutions. Thus, here we only describe a protocol for preparing samples to be measured by TEM. It is necessary to prepare a dispersion of the NPs in d.H_2O as the sample has to be dried (measurements are performed in vacuum) and salt crystallization can occur if the NPs are dispersed in buffer affecting the experiment. If the NP stock is dispersed in buffer, it is also possible to wash the sample directly on the grid.

Materials

TEM grid (the chosen material depends of the NP material and the specifics of the apparatus and manufacturer)

TEM instrument with imaging modality

NP stock dispersions (concentrations and nanomaterials tested are to be pre-determined by the experimenter)

1. Wash the grid with a suitable clean solvent as indicated by the supplier (it depends on the material of the grid).

IMPORTANT NOTE: Never touch the grid with hands but always use suitable tweezers.

2. Transfer the NP dispersion onto the grid by multiple depositions of 5-10 µl. After each deposition let the solvent evaporate before adding the following drop.

If it is necessary (i.e. if the NPs are dispersed in salt solutions) wash the grid with d.H_2O to eliminate the salts as this operation should not remove the NPs, which are adhered to the grid surface.

A rough calculation of the amount of NPs transferred to the grid should be done for evaluating the number of depositions necessary to reach the minimum amount of sample to perform a statistically significant measure.

3. Leave the grid to dry overnight, ideally under a hood and protected from dust.
4. Perform the measurement taking pictures of different areas on the grid.
5. For each grid (sample) several images are taken and saved. The images are analyzed with specific image software that allow extracting size information, thus a size-distribution can be determined.

IMPORTANT NOTE: To be statistically meaningful the size-distribution must be done on at least 100 NPs.

TEM size is often 10% smaller than the hydrodynamic size that also includes the hydration layer.

BASIC PROTOCOL 2 – CYTOTOXICITY ASSESSMENT OF NP TREATMENT

A crucial part of our nanotoxicity protocol is cytotoxicity assessment in mammalian cells, as due to their unique material composition, some nanoformulations can have harmful toxic effects in mammalian systems. Multiple factors can influence the extent of nanomaterial toxicity such as NP size, morphology, chemical structure and surface chemistry (Caballero-Diaz and Valcarcel Cases, 2016). A wide variety of conventional *in vitro* assays are available to assess nano-cytotoxicity, for example; 3-(4,5 dimethylthiazol)-2,5 diphenyltetrazolium bromide (MTT), which is a commonly used cytotoxicity assessment assay that has been successfully used to detect nanotoxicity (Gulati et al., 2010; Hussain et al., 2005; Park et al., 2010; Schubert et al., 2006; Webster et al., 2016; Yuan et al., 2010) and provides a simple, reproducible and reliable test set-up. In addition to MTT, nanotoxicity in mammalian cells can be evaluated by a variety of other cytotoxicity assessment methods including; 2′,7′-

Dichlorofluorescein (DFC) assay, proinflammatory cytokine ELISA, TUNEL, Trypan Blue Exclusion assay, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; MTS), CellTiter-Glo, adenosine triphosphate luminiscence, alamar blue (resazurin assay), neutral red staining, lactate dehydrogenase content analysis, phosphatidylserine translocation monitored by Annexin V staining, mitochondrial membrane potential and apoptotic protein level/activity, to name several.

Depending on their specific NPs and experimental conditions, users can select cytotoxicity methodologies to suit, as some nanoformulations can affect cytotoxicity readings by certain experimental approaches (Belyanskaya et al., 2007; Davoren et al., 2007; Hillegass et al., 2010; Monteiro-Riviere et al., 2009; Wang et al., 2011). Two or more cytotoxicity protocols need to be employed to ensure that the nanotoxicity assessment is robust, which ideally should test more than one of the following cytotoxicity assessment parameters; oxidative stress, cell death, cell viability and inflammatory response. Table i provides a list of conventional cytotoxicity assessment assays and which NPs are compatible with these methods. Here we describe a protocol that we have previously used for NP cytotoxicity assessment to analyse cell viability using two methods; MTT and Trypan Blue Exclusion assay (support protocol 2), and cell death by assessing apoptotic markers (support protocol 3).

Materials

Mammalian cell lines of choice (recommended a minimum of 3 should be used)

Liquid N_2 cryogenic cell storage Dewar flask (for long-term storage of cell stocks; Cole-Palmer)

Water bath (set to 37 ºC; Fisher Scientific; an anti-microbial agent should be added to the water tray to limit contamination)

GM containing supplements as required (e.g. foetal bovine serum, amino acids, antibiotics etc., as required depending on the chosen cell types. GM details for specific lines are provided by the supplier or in the scientific literature. All reagents must be cell culture grade)

70% ethanol (Sigma-Aldrich)

Class II biological safety cabinet (Monmouth Scientific)

Sterile, disposable cell culture plastic ware (including flasks, plates, tubes, tips etc. For adherent cells, flasks and plates must be cell culture grade)

Humidified 37 ºC, 5% CO_2 cell culture incubator (New Brunswick; an anti-microbial agent should be added to the water tray to limit contamination)

Inverted light microscope (Olympus)

Phosphate buffered saline (PBS; see recipe)

0.05% (w/v) Trypsin-EDTA solution (cell culture grade; Sigma-Aldrich)

Swing-out (bucket) centrifuge (Eppendorf)

Automated cell counter (e.g. Bio-Rad TC20^{TM}) or a Neubauer hemocytometer (Merck Millipore)

Mycoplasma testing kit (we use the EZ-PCR mycoplasma test kit; Gene Flow)

Multichannel pipette (Fisher Scientific)

NP exposure solution (concentrations and nanomaterials tested are to be pre-determined by the experimenter)

MTT solution (Sigma-Aldrich; prepared according to the manufacturer's instructions)

Dimethyl sulfoxide (DMSO; Sigma-Aldrich)

Sorensen's glycine buffer (see recipe)

Microplate spectrophotometer reader (SpectraMax)

1. Resuscitate mammalian cells from cryopreservation. Grow according to recommendations for the chosen cell lines, according to good lab practice (GLP). Correct handling and GLP for cell culturing involves the use of aseptic technique to avoid contamination of the cultures (Freshney, 2010). Furthermore, cells should be used at low passage numbers (<25) to avoid genetic drift and lines should be validated, and checked for contaminants prior to experimental use.

Three or more cell lines should be selected by the experimenter to assess nanotoxicity. The selection of these lines should be based upon the predicted exposure routes of the nanomaterial being assessed. For example, we have previously assessed iron oxide NP cytotoxicity in cell lines that represent possible exposure tissues in man, i.e. lung epithelium (A549), skin (SK-MEL-28) and kidney epithelium (MDCK), and that are easy to grow (Webster et al., 2016).

Supplementation of GM with antibiotics is optional. If it is used we recommend 100 µg/ml penicillin/streptomycin.

IMPORTANT NOTE: *GM is prepared in advance and can be used for several weeks if stored at 4 °C. It should be pre-warmed to 37 °C using a water bath prior to use on the cells to avoid cold shock. Water baths are a source of contamination in cell culture facilities and therefore should be regularly checked and cleaned, and an anti-microbial agent added to the water.*

IMPORTANT NOTE: *Maintenance and preparation of mammalian cell lines should be conducted in a class II biological safety cabinet and 70% ethanol used to sanitise all reagents and plastic ware used in the hood. All reagents must be prepared under aseptic conditions.*

IMPORTANT NOTE: *Like water baths, cell culture incubators represent another source of potential contamination. They too should be regularly checked, cleaned and a non-toxic anti-microbial added to the water tray.*

2. Trypsinise and seed cells at 4500 cells/well in a 96-well, flat-bottomed plate in triplicate (as a minimum for experimental replicates). Incubate cells overnight in cell culture incubator.

IMPORTANT POINT: Due to the edge effect on cell culture plates, conditions in the outer-most wells can lead to assay variability. We recommend not using the outer-most wells and rather only add GM or PBS to them.

For non-adherent, suspension cells, treated samples should be collected, spun down, resuspended in a fresh medium and treated with MTT solution.

3. Wash cells with PBS (enough to cover the monolayer) and add NPs at the desired concentration in GM at a volume of 150 µl/well. For the control wells add 150 µl/well of GM alone. Incubate cells for 72 hrs.

IMPORTANT NOTE: *Careful pipetting technique must be used whilst washing, removing and adding GM to the cells. For adherent cells disturbance of the monolayer can dramatically affect the assay results.*

4. Following incubation with the NPs, remove the treatment media and wash the cells twice with PBS. Prepare fresh media of 50 µl of MTT (2 mg/ml) in d.H_20, added to a total volume of 250 µl/well and incubate the plate for a further 4 hrs.

During this time the cells can be checked for the development of formazan crystals (formed through the reduction of tetrazolium salts), which appears as an intracellular purple precipitate.

5. Carefully remove the MTT solution to leave the insoluble formazan precipitate. Add 200 µl of DMSO/well and 25 µl of Sorensen's glycine buffer/well. Mix gently to resuspend the formazan crystals.

From this point onwards the experiment does not need to be conducted using aseptic technique.

IMPORTANT NOTE: *During mixing, avoid the production of air bubbles that could otherwise affect the optical absorbance readings.*

6. Remove the plate cover and measure the absorbance in each well at 570 nm wavelength using a microtitre plate reader for optical absorbance.

7. Calculate the percentage cell viability as a ratio of mean absorbance from the replicates with respect to the control treatments, using the following formula:

*% cell viability = (*I*sample/*I*control)*100 [where* I = *absorbance intensity]*.

SUPPORT PROTOCOL 2 – TRYPAN BLUE EXCLUSION ASSAY

As highlighted in Basic Protocol 2, >1 cytotoxicity assay should be employed to determine nanotoxicity in mammalian cells. Here we describe the use of trypan blue exclusion assay to support the findings from MTT analysis (see Basic Protocol 2). Trypan blue determines the number of live and dead cells depending of the principle that intact plasma membranes exclude the dye, whereas damaged/dead cells do not (Avelar-Freitas et al., 2014). Mammalian cells stocks are maintained and prepared using GLP as described above (basic protocol 2, point 1).

Materials

Mammalian cell lines of choice (recommended a minimum of 3 should be used)

See basic protocol 1 for a detailed list of equipment and reagents required for growing mammalian cell lines.

NP exposure solution (concentrations and nanomaterials tested are to be pre-determined by the experimenter)

0.4% trypan blue solution (Sigma-Aldrich)

1. Trypsinise and seed mammalian cells at 20000 cells/well in a 24-well, flat-bottomed plate in triplicate (as a minimum). Incubate cells overnight in a cell culture incubator.
2. Gently wash cells with PBS (enough to cover the monolayer) and add NPs at the desired concentration in GM at a volume of 500 µl/well. For the control wells add 500 µl/well of GM alone. Incubate cells for 72 hrs.
3. Following incubation with NPs, gently wash cells twice with PBS and use 100 µl/well trypsin/EDTA to detach cells from the well. Mix 10 µl of the cell suspension 1:1 with 0.4% trypan blue solution. Incubate for 2 min at room temperature.

Trypan blue should be stored in a dark bottle at room temperature and filtered with a 0.2 µM filter if used after prolonged storage.

4. Count the unstained (viable) and stained (non-viable) cells. Calculate cell viability using the following equations:

*% cell viability = (*unstained cells/total cells*)*100*

*% non-viable cells = (stained cells/total cells)*100*

SUPPORT PROTOCOL 3 – IMMUNOBLOTTING FOR APOPTOTIC MARKERS

Immunoblotting (or Western blotting) is a molecular technique used to detect proteins in a complex milieu. Following extraction from cells, proteins are separated (usually by sodium

dodecyl sulphate-polyacrylamide gel electrophoresis; SDS-PAGE) and then immunoblotted by transferring the proteins to a solid substrate and proteins of interest detected using antibodies targeted against them. Here we describe a protocol using immunoblotting to assess apoptotic cell death in response to NP treatment. A variety of markers can be used to assay apoptosis, should reduced cell numbers be detected in Basic Protocol 2/Support Protocol V (e.g. cleaved Caspase [3,8 and 9], Puma, Noxa and p7056K). Here we describe the use of cleaved Poly (ADP-ribose) polymerase-1 (PARP1) as a read-out of apoptosis. During this type of cell death, Caspase/protease-mediated cleavage of PARP1 in fragments of 89/24kDa is a useful and easily detectable apoptotic hallmark (Kaufmann et al., 1993). The basic protocol is adapted from immunoblot protocols used in our previous work (Jenei et al., 2009; Webster et al., 2016).

Materials

Mammalian cell lines of choice (recommended a minimum of 3 should be used)

See basic protocol 1 for a detailed list of equipment and reagents required for growing mammalian cell lines.

NP exposure solution (concentrations and nanomaterials tested are to be pre-determined by the experimenter)

A cytotoxic agent that can be used as a positive control in the cell lines of choice (e.g. cisplatin; this agent and dose should be pre-determined for each cell line selected).

PBS (see recipe); 0.1-0.5 L needs to be cooled to 4^{o}C

Protein extraction buffer (containing protease inhibitors; see recipe; cooled to 4^{o}C)

Plastic cell scrapers (Thermo Fisher Scientific)

1.5 ml Eppendorf microcentrifuge tubes (Thermo Fisher Scientific)

Sonicator (Diagenode™ Bioruptor® Pico Ultrasonicator; Thermo Fisher Scientific)

-20^{o}C freezer

Pierce^{TM} BCA Protein Assay kit (Thermo Fisher Scientific)

UV-Vis Spectrophotometer (Orion™ AquaMate 8000; Thermo Scientific)

Dithiothreitol (DTT; Sigma-Aldrich)

SDS (Sigma-Aldrich)

Loading buffer (see recipe)

Dry block heating system

Tris-HCl buffer (see recipe; Sigma-Aldrich)

40% acrylamide/bisacrylamide (Sigma-Aldrich)

Ammonium persulfate (APS; Sigma-Aldrich)

>99.5% tetramethylethylenediamine (TEMED; Sigma-Aldrich)

Mini gel tank and associated casting plates, combs etc. (Mini-PROTEAN® Tetra Vertical Electrophoresis Cell ; Bio-Rad)

Running buffer (10X; see recipe)

Protein Molecular Weight Standards (range = 6500-205,000 Daltons; Thermo Fisher Scientific)

Gel-loading tips (range 0.5–200 µL; Thermo Fisher Scientific)

Universal Power Supply (PowerPac™ ; Bio-Rad)

Polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific) or nitrocellulose membrane (Thermo Fisher Scientific)

Bent-tip stainless-steel forceps (Thermo Fisher Scientific)

10X transfer buffer (see recipe)

Sponge pad for blotting (Invitrogen)

Tris-buffered saline/Tween20 (TBST; see recipe)

Shaker plate/roller

Ponceau S solution (Sigma-Aldrich)

Blocking solution (see recipe)

Rabbit anti-PARP-1 antibody (sc-7150; Santa Cruz Biotechnology)

Anti-rabbit Horseradish-peroxidase (HRP)-conjugated secondary antibody (#7074; Cell Signalling Technology)

ECL™ Western blotting detection reagent (GE Healthcare)

ChemiDoc™ XRS+ system (Bio-Rad)

Image analysis software (ChemiDoc Touch, Bio-Rad)

Mouse anti-α-tubulin (DM1A; Cell Signalling Technology)

Anti-mouse Horseradish-peroxidase (HRP)-conjugated secondary antibody (#7076, Cell Signalling Technology)

Protein preparation from mammalian cells

1. Trypsinise and seed mammalian cells at $1x10^6$ cells/10 cm diameter petri dish (cell culture grade) and incubate cells overnight in a cell culture incubator.

2. Gently wash cells with PBS (enough to cover the monolayer) and add NP/control treatments at the desired concentration in GM, at a volume of 5-10 ml/plate. Incubate cells for 72 hrs.

A positive control (pro-apoptotic drug) treatment should be used to ensure the detection of apoptosis in the cell type of choice.

3. Remove the GM and wash cells twice in ice-cold PBS (enough to cover the monolayer). Remove PBS and add 300 µl/plate ice-cold lysis buffer. Using a cell scraper (chilled to 4^{o}C), scrape the cells off the dish then gently transfer the resulting lysate in a pre-cooled microfuge tube.

IMPORTANT POINT: *This step should be carried out on ice. From this step onwards keep all fractions and reagents used on ice throughout.*

4. Sonicate sample for 15-30 secs, typically 20 to 50 kHz.

At this frequency, sonication ensures complete cell lysis and shears the DNA to reduce sample viscosity.

5. Centrifuge lysate at 4^{o}C for 20 min at 16000*g*. Gently aspirate the supernatant containing the protein extract and store in fresh cold tubes.

At this point samples can be stored as aliquots at -20 ^{o}C. Avoid repeated freeze-thawing as this can reduce sample integrity.

6. Determine protein concentration using the Pierce^{TM} BCA Protein Assay kit (Thermo Fisher Scientific) according to the manufacturer's protocol, or using a similar technique (e.g. the Bradford assay;(Bradford, 1976).

Perform SDS-PAGE

7. Prepare 10-25 µg of total protein by adding DTT at a final concentration of 0.1 M, 1% SDS in loading buffer (4X stock volume) to a total volume of 10-25 µl/sample. Denature samples at 90 ^{o}C for 10 min.

DTT functions as a reducing agent to reduce disulphide bridges, whilst SDS functions as an anionic denaturing detergent.

IMPORTANT NOTE: *Wear gloves at all times when handling SDS-PAGE gels, as acrylamide is a potent, cumulative neurotoxin.*

8. Assemble the SDS-PAGE gel tank system and add 1 X running buffer to the top. Carefully load the protein in the desired sequence and load protein markers according to the manufacturer's instructions.

Alternative gel tank systems are available from different manufacturers, so follow the assembly instructions for different apparatus accordingly.

Prepare the gel the same day or the day before (storing overnight in running buffer at 4 ^{o}C). Alternatively pre-cast gels can be purchased.

Careful loading is critical to avoid sample spill over between adjacent gel lanes. We recommend using gel-loading tips to prevent this.

9. Using gel electrophoresis, separate the proteins in a 12% SDS-PAGE resolving gel, overlaid with a 5% stacking gel (Table ii). Run protein separation at 90 V through the stacking gel and 120 V through the resolving gel.

10. Once the proteins are fully resolved, dismantle the SDS-PAGE apparatus. Carefully remove the gels from the casting plates, remove the stacking gel and discard. Keep the resolving gel moist in transfer buffer, whilst preparing for immunoblotting.

Perform immunoblotting

11. Pre-soak a nitrocellulose transfer membrane in 1 X transfer buffer for 5 min.

If using a PVDF membrane, pre-soak in 100% methanol.

Membrane handling should be kept to a minimum and only use membrane forceps when manipulating to reduce background staining.

12. Prepare the transfer sandwich as previously described (Gallagher et al., 2008). Briefly, sandwich the gel and membrane between layers of pre-soaked filter paper/blotting sponges (in 1 X transfer buffer) in a transfer cassette, ensuring tight contact between the gel and membrane. For tank blotting, assemble the transfer sandwich in the gel tank and perform protein transfer in 1 X transfer buffer at 4 ^{o}C, ensuring the membrane faces the anode.

IMPORTANT POINT: *Avoid air bubbles between the gel and membrane as this can lead to poor protein transfer. Using a clean pipette to roll over the membrane when assembling the transfer sandwich can easily remove bubbles.*

Alternatively protein transfer can be done using semi-dry blotting apparatus. These systems should be used according to the manufacturer's instructions.

13. Once protein transfer is complete, carefully dismantle the transfer sandwich. Wash the membrane twice in 1 X TBST (enough to cover the membrane) for 5 min on a shaker plate/roller.

14. Stain the membrane with Ponceau S solution (enough to cover the membrane) for 1 min, to visualise proteins and ensure complete transfer (protein bands will stain red). Then wash the stain away with $d.H_20$ several times with agitation until all the Ponceau S solution is removed from the membrane.

15. Block the membrane for 1-2 h at room temperature with agitation in blocking solution (containing 5% milk; enough to cover the membrane).

5% BSA can also be used as a blocking reagent and for alternative antibodies to the ones suggested here, should be used as recommended for individual antibody clones.

16. Dilute the anti-PARP-1 antibody in enough blocking solution to cover the membrane and incubate with the membrane overnight at 4 ^{o}C with constant gentle agitation.

We standardly use a 1:200 dilution, but this will require optimisation for individual cell types to determine the optimal antibody/protein ratio.

17. Wash the membrane three times in 1 X TBST for 10 min each at room temperature with constant agitation.

This step is important to remove any unbound antibody.

18. Add the cognate secondary antibody diluted in blocking solution for 1 h at room temperature with gentle agitation.

Use the secondary antibody at a minimal dilution of 1:2500, although this will require optimisation for the cell types used.

19. Repeat step 17. Incubate the membrane with ECL reagent (according to the manufacturer's instructions) and detect the chemiluminescent signal using the desired imaging system (e.g. the ChemiDoc^{TM} XRS+ system; Bio-Rad). Use image analysis software to analyse protein band intensity.

X-ray film (with/without automated developing) is also a commonly used method for signal detection.

20. Rinse membrane in methanol and then repeat step 17. Dilute the anti-α-tubulin antibody in enough blocking solution to cover the membrane and incubate with the membrane overnight at 4 ^{o}C with constant gentle agitation.

Detection of α-tubulin in the cells is used as a loading control. The choice of a loading control can be modified depending on the cell type used and the size of the protein(s) of interest being detected by immunoblotting.

21. Repeat steps 17-19.

Determining the ratio between the cleaved PARP-1 (89 kDa) and full-length PARP-1 (116 kDa) bands relative to the gel loading control, can be used as a readout for caspase-mediated apoptosis.

BASIC PROTOCOL 3 – *X. LAEVIS* PHENOTYPIC ABNORMALITY ASSAY FOR NANOTOXICITY ASSESSMENT

This protocol is designed to be used in parallel with cell-based cytotoxicity assays as part of an integrated toxicity assessment in order to obtain a complete safety profile of a novel NP (Fig. i). *X. laevis* is an ideal model organism to be used for comparatively high-throughput screening (Tomlinson et al., 2009) and has been used as a toxicity model in the frog

teratogenesis assay-*Xenopus* (or FETAX assay) for drugs in their early stages of drug safety evaluation (Leconte and Mouche, 2013). This is largely due to *X. laevis* being a relatively inexpensive and rapid model that that can be easily scaled-up as a large number of embryos can be produced. *X. laevis* embryos develop externally, making them an easily accessible system for exposure to NPs. Previous work has shown that this methodology allows both external NP exposure and internal exposure to key internal organs for assessing potential toxicity (Webster et al., 2016). Briefly, *X. laevis* embryos are exposed to a NP-containing incubation solution over a desired developmental period that can be adapted depending on the specific aims of the nanotoxicity assessment protocol.

Materials

Nieuwkoop and Faber (NF) stage 1 *X. laevis* embryos (see Support Protocol 4)

MMR solution (see recipe)

Pasteur pipette (we recommend glass. Whole embryos are too large to fit into a standard pipette, therefore mark the end with a diamond pen, break off cleanly and fire the end briefly to melt any sharp edges. Alternatively, if desired, plastic Pasteur pipettes can be used with the end removed)

10 cm^2 Petri dish (Fisher Scientific)

Culture incubator (set to desired temperature; see below for details)

Stereomicroscope with two-armed fibre optic illuminator to allow the angle of illumination to be easily adjusted

Dumont #5 forceps (stainless steel; Sigma-Aldrich). These are ultrafine and can be used for carefully manipulating embryos throughout the described protocol.

24-well culture plate (non-cell culture grade; Fisher Scientific)

NP exposure solution (concentrations and nanomaterials tested are to be pre-determined by the experimenter)

Ethyl 3-aminobenzoate methanesulfonate (0.6 mg/ml; Fluka)

MEMFA fixative (see recipe)

Phosphate-buffered Saline (PBS) and PBST (see recipes)

3 cm^2 Petri dish (Fisher Scientific)

Agarose gel (2% [w/v]; Sigma-Aldrich; see recipe)

Long-handled scalpel (10A blades)

Light microscope with charge coupled-device (CCD) digital camera for whole-mount imaging of embryos

Methanol (analytical grade; Sigma-Aldrich), 25%, 50%, 75%, 100% [v/w] in PBS

Glass vials with screw caps (3.5 ml; SGL)

Parafilm M™ wrapping film (Fisher Scientific)

-20 °C freezer

1. Harvest NF stage 1 *X. laevis* embryos (see Support Protocol 4) and incubate between 12–23°C until at required developmental stage (Fig. iii). During the incubation times it is important to regularly observe the embryos (at least twice daily or more at early stages) to remove any dead embryos and ensure the correct NF stage has been reached.

Developmental times of embryos are dependent on incubation temperature and culturing them at differing temperatures can speed or slow development. Typically, after incubation at 23°C, embryos are NF stage 4 after ~ 2 h, NF stage 15 after ~ 17 h, and NF stage 38 after incubation for ~ 2 days 5 h.

IMPORTANT NOTE: *Bacteria grow well at the higher incubation temperatures so embryos cultured between 18-25 °C should be regularly monitored and washed twice*

daily. To avoid this problem, the 0.1X MMR culture media of later stage embryos (NF stage 23 onwards) can be supplemented with 25 µg/ml of gentamicin.

2. In a 24-well plate, add 200 µl of NPs in 0.1 X MMR solution to each well at a concentration that is 10 X higher than that of the desired final concentration. For the control wells, add 200 µl 0.1 X MMR alone.

3. At the required NF stage, select 5 healthy embryos and transfer using a volume of 1800 µl 0.1 X MMR using a glass Pasteur pipette into one well of a 24-well plate. Repeat until the wells for each of the desired NP concentrations (along with the control wells) contain 5 embryos to a final volume of 2 ml. Incubate at the same temperature that the embryos were initially developed at.

4. Continue to incubate *X. laevis* embryos until they have reached the desired end stage (Fig. iii).

Again it is important that the embryos are checked several times a day to identify any dead ones and to assess developmental progress. Dead embryos should be removed from the well during this incubation period and the number of dead recorded.

5. Make a note of any dead *X. laevis* embryos at the end of the incubation time. Wash embryos with 0.1 X MMR and using a Pasteur pipette, gently transfer to a new 24-well plate containing 1 ml of 0.6 mg/ml ethyl 3-aminobenzoate methanesulfonate salt to anesthetise the embryos. Incubate for 20 min at room temperature to ensure embryos are fully anesthetised prior to fixing (Sherwood et al., 2008; Webster et al., 2016).

A variety of nanomaterials are synthesised for use as fluorescent bioimaging tools (Wolfbeis, 2015). If such fluorescent NPs are being tested using this protocol (e.g. metal chalcogenide quantum dots [QDs]) they can be detected in the embryos

using live whole-mount fluorescent imaging at this stage in the protocol (Webster et al., 2016); see support protocol 5).

6. Wash away the anaesthetic solution with several rinses of 0.1 X MMR before fixing the embryos with MEMFA for 1 h at room temperature or overnight at 4^{o}C.

IMPORTANT NOTE: *Fresh MEMFA should be prepared for each experiment.*

If the embryos are going to be used for transmission electron microscopy (TEM; which can be used to determine exposure to non-fluorescent NPs) then MEMFA should not be used for embryo fixation. Rather an alternative fixing protocol provides improved ultrastructural analysis of X. laevis *embryos by TEM (see alternate protocol 1).*

7. Following fixation, aspirate off as much MEMFA as possible and wash embryos twice with excess PBST.

8. Whole-mount images of the embryos should now be taken to assist with phenotypic scoring. To do this, prepare a 2% (w/v) agarose gel by heating 100 mg agarose in 5 ml PBS until all the agarose has dissolved. Then pour the 2% agarose into the bottom of a 10 cm^3 culture dish and leave to cool and set (should take approximately 30 minutes).

Agarose gel-containing imaging wells (as described above) can be prepared in advance of the experiment and stored at 4 ^{o}C prior to use.

Once set, a small indentation/notch can be made in the agarose gel using a scalpel to help position the embryos for imaging.

9. Pour a small layer of PBS over the agarose gel. Gently transfer the *X. laevis* embryos into the agarose gel-containing dish using a Pasteur pipette and use this as a platform for imaging.

The PBS should cover the embryos so that they remain hydrated, but not be in excess such that it is difficult to retain the embryos in the desired position for imaging.

10. Observe each embryo using a light microscope and rank for phenotypic abnormalities (Table iii). Calculate phenotypic abnormality; the number of malformed larvae as a percentage of the total number at the beginning of the experiment. Likewise percentage mortality should be calculated in the same way.

Common abnormalities induced by NP exposure include loss of melanocytes, blistering, edema, tail loss, bent spine, degradation of tissue, developmental delay, eye deformities, and stunted growth (Webster et al., 2016); Table iii).

Exposure should be confirmed of NPs that do not produce notable nanotoxicity as scored in this phenotypic abnormality assay. If the NP is fluorescent this can be done as described in point 5 (see support protocol 5), but if not we propose that transmission electron microscopy (TEM) imaging of X. laevis tissue will facilitate confirmation of NP uptake in the embryos (see alternate protocol 1).

11. Following scoring, dehydrate the embryos for long-term storage. To do this, transfer the embryos into glass vials using a Pasteur pipette. Gently aspirate the PBST and replace with 25% methanol in PBS for 5 min, completely immersing all embryos in the glass vial.

12. Then aspirate the 25% methanol and immerse the embryos in 50% methanol. Repeat this step with 75% methanol and finally 100% (with 5 min between each concentration).

If required, embryos can be rehydrated for further analysis by reversing steps 12 and 11.

13. After dehydration, *X. laevis* embryos can be stored long term in 100% methanol at -20^{o}C. Finally, seal the glass vial cap with Parafilm for long-term storage at -20^{o}C.

SUPPORT PROTOCOL 4 – HARVESTING *X. LAEVIS* EMBRYOS

X. laevis have been used as model organisms for biological research for decades, particularly as developmental vertebrate systems. As a result, detailed methodologies have been devised to obtain and work with *X. laevis* embryos (Sive et al., 2000). Ethical legislation and considerations must be in place when working with adult *X. laevis* frogs, the specific requirements of which will be dependent upon geographical and institutional location. This is not only a legal requirement in many countries, but such ethical considerations will also assist with maintaining a well cared for population of adult frogs for generating healthy embryos. You will need access to an aquarium facility for holding *X. laevis* colonies, where males and females should be housed in separate tanks. The following protocol describes the steps required to collect eggs and conduct fertilisations in order to obtain *X. laevis* embryos for nanotoxicity assessment (see Basic Protocol 3).

Materials

Female *X. laevis* adults (2 or more)

Pregnant mare serum gonadotrophin (PMSG; Intervet)

25-gauge (25G; BD Biosciences) needle and 1 ml syringe (Fisher Scientific)

Non-textured, powder-free gloves (Fisher Scientific)

Human chorionic gonadotrophin (hCG; Intervet)

Culture incubator (set to 17^{o}C)

10 cm^{2} Petri dish (Fisher Scientific)

MMR (see recipe)

1 male *X. laevis* adult

0.05% Ethyl 3-aminobenzoate methane sulfonate (Fluka)

Surgical equipment including scalpels, forceps and curved scissors

Testis buffer (see recipe)

-20°C freezer

d.H_2O

Cysteine de-jellying solution (see recipe)

Glass beaker

1. Prime female *X. laevis* with an injection of 100 units of PMSG into the dorsal lymph sac 5–7 days before requiring embryos.

We recommend priming and inducing ovulation in >1 female, in case egg yield and quality is not good, as this can vary greatly between individual animals.

2. Isolate testes from an adult male *X. laevis* by first anesthetising him by submersion in 0.05% Ethyl 3-aminobenzoate methanesulfonate for a minimum of two hours. Remove the testes by exposing the abdominal cavity and drawing out the fat body with forceps. The testes lie at the base of the fat body and can be identified as white, oval shaped organs covered in a fine network of capillaries. Remove both testes and store in testes buffer at 4 °C for up to 14 days post-isolation.

IMPORTANT NOTE: *The male should be dead due to the overdose of anaesthetic. Confirm no reaction by pinching the toes before starting the surgery. Snip the heart prior to-, and freeze the sacrificed male, post- isolation of the testes.*

3. Induce ovulation by injection of 250 units hCG into each of the dorsal lymph sacs (500 units total) using a 25G needle. Incubate induced females at 17°C.

The dorsal lymph sac is located directly rostral to the hind limbs. It can be located between the lateral line that appears as 'stich' marks on the adult's skin and the spine.

IMPORTANT NOTE: *The skin covering the dorsal lymph sac is loose and therefore it is straightforward to insert the needle subcutaneously and inject the hCG, however it is crucial not to penetrate too deeply into the muscle.*

4. After 12-14 h the females should be ready to lay, which can be seen as the cloaca will appear red and swollen (due to the oocytes collecting in a sac close to this region). Gently 'squeeze' the abdomen of the female *X. laevis* to encourage egg release into a 10 cm^2 Petri dish containing 0.1 X MMR (enough to cover the eggs). This is done by very gently applying lateral/vertical pressure to the lower abdomen.

IMPORTANT NOTE: *Eggs should be fertilised immediately when collected in this manner. From this point onwards in the protocol it is critical to progress as rapidly as possible through the remaining steps, this helps ensure quality of the resulting embryos.*

As an alternative to 'squeezing', eggs can be collected passively by allowing females to lay in 1X MMR, where eggs will be viable for fertilisation for up to 8 h post-laying.

5. Fertilise the harvested eggs by cutting off a small piece of one testis (<25%) and homogenise the testis section using a scalpel blade and forceps. Add 1ml 1X MMR to the mashed up testis piece. Mix the testis slurry well with the eggs across the entire dish to promote fertilisation. Leave for 5 minutes then flood the dish with 0.1X MMR and leave for 20-30 minutes.

6. Incubate the eggs at 17 ^{o}C and monitor regularly for successful fertilisation. The first sign is a cortical contraction of the animal pole approximately 5 min post-fertilisation. However, by 15-30 min fertilised eggs will reorient such that the animal pole faces up, which is the most reliable sign that fertilisation has been successful.

The release of cortical granules into the space between the fertilised egg and the vitelline membrane blocks polyspermy and causes the eggs to turn with their

membranes according to gravity, with their pigmented animal poles facing up. At this point fertilised eggs will be much firmer than unfertilised ones, so it is easy to tell if the fertilisation has been successful or not by 30 min post-fertilisation.

7. Continue to incubate the fertilised eggs at 17 ºC for another 1-2 h. Upon entering the first cell cycle, cortical rotation occurs, which is required for formation of dorsal tissues and usually occurs within 2 h of fertilisation at 17 ºC.

IMPORTANT NOTE: *Do not disturb the embryos during this incubation period too much as it can interfere with correct dorso-ventral patterning. For example, shaking the embryos during this time is known to produce spontaneous secondary axis formation through microtubule reorientation.*

8. *X. laevis* embryos are surrounded by a thick layer of protective jelly that must be removed prior to further experimentation. Ideally this should be done after cortical rotation to reduce the likelihood of developmental defects (see point 7). In a glass beaker, gently swirl the embryos in 2% cysteine (w/v) de-jellying solution until they pack closely together.

The time required for this step can vary depending on differences between embryo batches, however it should normally take around 5 min and no longer than 10 min.

IMPORTANT NOTE: *The de-jellying solution needs to be made fresh on the day of use and used at room temperature.*

IMPORTANT NOTE: *Do not over-treat as this can lead to developmental defects and can contribute to poor embryo quality.*

9. Remove cysteine solution and wash the eggs several times with distilled water (>5 washes) followed by several washes with 0.1 x MMR. Embryos are then reared in 0.1 X MMR, ready for further experimental procedures.

SUPPORT PROTOCOL 5 – WHOLE-MOUNT IMAGING OF *X. LAEVIS* EMBRYOS FOR FLOUORESCENT NP UPTAKE

This protocol can be used to investigate internalisation of fluorescent NPs in *X. laevis* embryos. We have previously demonstrated that this protocol works well using 20 nm fluorescent carboxylate-modified NPs (PS-COOH; Molecular Probes FluoSphere beads®; Thermo Fisher Scientific®; catalog #F8887), thus we propose that these NPs offer a useful positive control for NF stage 45 embryos, exposed to 10^{15} NP/ml from NF stage 38 (Webster et al., 2016).

Materials

3 cm^2 Petri dish (Fisher Scientific)

Agarose gel (2% [w/v]; Sigma-Aldrich; see recipe)

Long-handled scalpel (10A blades)

Tadpole stage *X. laevis* embryos (from NF stage 38 onwards; see Fig. iii), anesthetised and pre-exposed to florescent NPs (see point 5, Basic Protocol 3 for details). As described above, 20 nm PS-COOH NPs should be used as a positive control.

Glass Pasteur pipette (prepared as described in Basic Protocol 3)

Dumont #5 forceps (stainless steel; Sigma-Aldrich). These are ultrafine and can be used for carefully manipulating embryos throughout the described protocol.

PBS (see recipe)

Fluorescent microscope with CCD digital camera

1. Prepare agarose imaging plates for whole-mount *X. laevis* embryos as described in point 8, Basic Protocol 3.

2. Pour a small layer of PBS over the agarose gel and gently transfer the *X. laevis* embryos into the agarose gel-containing imaging plate (see point 9, Basic Protocol 3 for details).

3. Image the embryos using a fluorescent microscope according to the emission filter required to excite the NPs being tested.

For the PS-COOH NPs, an emission filter of 509-547 nm should be used. The fluorescence from these NPs will appear bright throughout the embryo (Webster et al., 2016).

4. Time-lapse images (with time-frame stills of 0.7 seconds) can be used to monitor fluorescent NPs traveling through the vasculature of the *X. leavis* embryos, which is particularly clear in the embryonic intersomitic blood vessels (Webster et al., 2016).

ALTERNATE PROTOCOL 1 – TEM IMAGING OF *X. LAEVIS* EMBRYO SECTIONS FOR NP UPTAKE

Support protocol 5 cannot be used to confirm uptake of non-fluorescent NPs in *X. laevis* embryos and for this reason such NPs require an alternative procedure to ensure embryo exposure to these nanomaterials. Electron microscopic techniques facilitate high-resolution visualization of NPs in tissues and in particular TEM has been used for a long-time in NP research. Due to the complexity of sample preparation, imaging and interpretation of ultrastructual NP localisation within tissues, and that the infrastructure required for TEM analysis is often housed in centralised facilities, where possible it is pertinent to seek advice about TEM experimental design with expert staff within such core facilities. This will assist with optimisation of advanced TEM imaging for specific nanomaterials, but here we describe

a protocol that is suitable for preparing high-quality *X. laevis* embryo sections that is suitable at least for imaging iron oxide core NPs (Webster et al., 2016). The processes of fixing, embedding and sectioning *X. laevis* embryos for TEM is based on a previously described method developed for imaging carbon NPs *in vivo* (Bacchetta et al., 2012).

Materials

Tadpole stage *X. laevis* embryos (from NF stage 38 onwards; see Fig. iii), anesthetised and pre-exposed to NPs (see point 5, Basic Protocol 3 for details).

Glass Pasteur pipette (prepared as described in Basic Protocol 3)

MMR (see recipe)

TEM fixing buffer (see recipe)

Osmium tetroxide (OsO_4; Sigma-Aldrich), 1% [v/w] in PBS

Methanol (analytical grade; Sigma-Aldrich), 25%, 50%, 75%, 100% [v/w] in PBS

Propylene oxide resin (TAAB Laboratories Equipment Ltd.)

Incubator (set to 60 ^{o}C)

Microtome (Reichert Ultracut E)

Carbon-coated 300 μM mesh copper grids (Agar Scientific)

TEM instrument with imaging modality

1. Immerse *X. laevis* embryos in 0.6 mg/ml ethyl 3-aminobenzoate methanesulfonate salt for 20 min at room temperature to anesthetise.

2. Wash away the anaesthetic solution with several rinses of 0.1 X MMR and fix the embryos in TEM fixing buffer (enough to immerse the embryos) for 1 h at room temperature. During this time replace the TEM fix twice with fresh buffer.

3. Post-fix the embryos in 1% OsO_4 for 1.5 h at 4 °C.

This step is needed to increase the electron density in lipids and proteins.

4. Dehydrate the fixed embryos in a decreasing concentration of methanol, as described for point 12 of basic protocol 3.

5. Once dehydrated, wash the embryos in 75% propylene oxide resin and leave in 100% pure resin overnight.

6. Submerse embryos in fresh resin and then polymerise at 60 °C for 48 h.

7. Using a microtome, cut semi-thin 1 μm sections of the embryos.

Cut in an anterior to posterior direction to produce transverse sections along the entire embryo. Analyse all tissues across the anterior-posterior axis as the location of the NPs will depend upon the biodistribution of specific nanomaterials within X. laevis embryos.

Ultrathin sections (~50 nm) can also be used if required for NP detection.

8. Mount the sections in onto carbon-coated 300 μm mesh copper grids.

9. Image sections using a TEM according to the settings required for the instrument.

As an example, we have successfully used a Tecnai™ 20 TEM (FEI; Thermo Fisher Scientific) with AMT cameras, operating at an acceleration voltage of 200 kV to image iron oxide core NPs (Webster et al., 2016). Likewise carbon NPs have been successfully imaged in X. laevis *embryos using a Zeiss LEO 912ab Energy Filtering TEM at 80 kV (Bacchetta et al., 2012).*

REAGENTS AND SOLUTIONS

General laboratory reagents are supplied by Sigma-Aldrich. Use d.H_20 in the following recipes (unless otherwise stated):

Agarose gel

100 mg agarose

5 ml PBS

Blocking solution

5 g BSA

100 ml 1 TBST

Blocking Buffer:

7.5 g nonfat dry milk

15 ml 10X TBS

0.15 ml Tween-20 (100%)

Final volume 150 ml

Cysteine solution

3 g cysteine

100 ml 0.1 x MMR

Adjust to pH 7.8 with 10 M NaOH

4X Loading buffer

3 mL of 1 M DTT

1.5 mL of 1 M pH 6.8 Tris-HCl

0.6 g of SDS

2.4 mL of glycerol

0.03 g of bromophenol blue

Final volume to 7.5 ml. Store at -20°C

Lysis buffer

50 mM Tris-HCl

1% Triton X-100

150 mM NaCl

Final volume to 200 ml in PBS. Adjust pH to 7.4.

MMR

100 mM NaCl

2 mM KCl

1 mM $MgCl_2$

2 mM $CaCl_2$

5 mM HEPES (pH 7.6)

Adjust to pH 7.4.

MEMFA

i) 10 X MEM salts *(autoclave and store in the dark)*

1 M MOPS

20 mM EGTA

10 mM $MgSO_4$

5 mM HEPES (pH 7.6)

Adjust to pH 7.4 with NaOH pellets. Dilute in d.H_2O for a 1 X working solution.

i) 1 X MEMFA

3.7% formaldehyde

1 X MEM salts

PBS/PBST

i) 10 X PBS

1.4 M NaCl

26.8 mM KCl

100 mM Na_2HPO_4

17.6 mM KH_2PO_4

Adjust to pH 7.4 with HCl. Dilute in d.H_2O for a 1 X working solution

ii) 1 X PBST

0.1% Tween-20

1 X PBS

Ponceau stain

0.2 g Ponceau S

5 ml glacial acetic acid

Final volume to 100 ml

10 X Running buffer

30.2 g Tris-base (25 mM)

144 g Glycine (190 mM)

0.1% SDS

Final volume to 1 L. Adjust to pH 8.3

Sorensen's glycine buffer

121 g Tris Base

28.55 ml Acetic Acid

50 ml, 0.5 M EDTA

Final volume to 500 ml. Adjust to pH 8.0

TBST

24.23 g Tris-HCl

80.6 g NaCl

0.1% Tween-20

Final volume to 1 L. Adjust to pH 7.6. Dilute in d.H_2O for a 1 X working solution. Add 0.1% Tween-20.

TEM fixing buffer

4% paraformaldehyde

2% glutaraldehyde

0.1M sodium cacodylate buffer (4.28 g sodium cacodylate in 200 ml d.H_2O)

Adjust to pH 7.4

Testis buffer *(in 1 X MMR)*

80% Foetal Calf Serum

50 µg/ml gentamycin-sulfate

10 X Transfer buffer

30.2 g Tris-base (25 mM),

144 g Glycine (190 mM)

0.1% SDS

Volume to 1 L. Adjust to pH 8.3

10 X Tris-HCl buffer

61 g Trizma Base

Volume to 1 L. Adjust to pH 7.6 using HCl

COMMENTARY

Background Information

Here we have described the use of non-specialist cytotoxicity testing protocols in combination with a *X. laevis* embryonic phenotypic assay for nanotoxicity assessment. Specifically, testing well characterised nanomaterials at the physico-chemical level (Basic protocol 1) with standard cytotoxicity assessment (Basic protocol 2) and using this in combination with the *X. laevis* embryonic phenotypic assay (Basic protocol 3), can bridge the gap between conventional *in vitro* (cell culture models) and *in vivo* (mammalian systems) nanotoxicity assessment (Webster et al., 2016). We have shown that direct comparison of the cytotoxicity and *X. laevis* data can provided a logical ranking system to generate an overall hazard score for NPs (Webster et al., 2016). Briefly, a simple scoring system ranging from 0-2 can distinguish hazard score, where NPs score 0 when the percentage of cell viability and healthy *X. laevis* embryos is >76%, 1 when this percentage ranges from 50-75% and 2 when it is <50%. From these criteria only NPs that score 0 in all nanotoxicity assessment protocols should progress to further toxicity assessment in mammalian models (Fig. i). This approach can reduce false negatives that could otherwise be generated from cell-based assays used in

isolation. Thus, only NPs that produce no-to-low toxicity assessment in the described protocol progress to further evaluation in mammalian systems, thereby reducing investment in time and money spent on more costly rodent models, which is important given the year on year increase in development of nanotherapeutics. Overall, this protocol provides biomedical researchers with nanotoxicity assessment at early stage in nanotherapeutic design to quickly and easily identify nanomaterials that require additional modifications for improved safety, prior to mammalian testing (Fig. i).

Critical Parameters and Troubleshooting

There are several critical parameters that will affect successful outcome of the described protocol and therefore must be considered by users. These parameters include the following:

Dosing and storage of NPs.

The most suitable conditions of NP storage depend on the type of material from which the NPs are composed of. It is not possible to state general conditions. The chosen medium should guarantee stability of the NPs over time. If the material is not sensitive to low temperature it is suggested to store stock solutions in the fridge, mostly if they contain organic/biological moieties to avoid degradation. Before making any measurements it is also necessary to check the stock solution in terms of homogeneity in order to guarantee the right evaluation of the dose. Often, NP dispersions can be affected by flocculation over time, if flocculation is reversible, this process does not represent a problem. It is only necessary to re-disperse the sediment in the dispersion through simple shaking and/or 5-10’ sonication of the NP dispersion before the measurement or preparation of the samples.

Cell culture considerations

There are several important considerations when conducting cytotoxicity analysis for NP testing. The first is to select cell types (3 or more) that best model the exposure route(s) and target organ(s) of the nanomaterial of interest. Next, the appropriate methodology must be selected that can accurately assess cytotoxicity of the NP of interest without the development of false-negatives/-positives, which is important to carefully consider because not all nanomaterials are compatible with commonly employed methods. For example, MTT (the method described here; Basic protocol 2) although being easy, quick and readily affordable, is not compatible with several types of NPs. Wang Yu and Wickliffe, 2011 indicated that titanium oxide nanoparticle (nano-TiO2) induces superoxide formation in mammalian cells that reduces tetrazolium salts and produces the absorbant formazan end products (Wang et al., 2011). Monteiro-Riviere, Inman and Zhang, 2009 showed that single-walled carbon nanotubes SWCNT and carbon black CB alone (absence of cells) interact with the MTT to cleave the tetrazolium ring and lead to a false positive reaction (Monteiro-Riviere et al., 2009). Whilst Belyanskaya et al., 2007 found that sodium dodecyl sulfate-suspended SWCNTs interfere more with MTT assay than polyoxyethylene sorbitan monooleate-suspended SWCNTs (Belyanskaya et al., 2007). Table i lists which types of NP-based materials have previously been demonstrated to be compatible with commonly employed cytotoxicity assays. Finally, it is essential to use GLP when conducting *in vitro* cell work; including cell line validation, equipment validation/maintenance, mycoplasma contamination testing, employment of strict aseptic technique and using low-passage cell culture are all critical in obtaining high-quality, reproducible cytotoxicity data.

Immunoblotting considerations

Immunoblotting is a simple molecular procedure for the quantitative detection of proteins in cells/tissues. Here we describe a protocol to detect apoptotic markers in response

to NP-induced cytotoxicity (Support protocol 3). Despite its simplicity, an array of problems can be encountered that require troubleshooting to prevent unexpected results and a comprehensive description of effective immunoblotting troubleshooting has previously been provided (Mahmood and Yang, 2012). Briefly, use fresh protein sample using lysis buffer containing phosphatase inhibitors to prevent sample degradation and ensure the transfer sandwich is effectively prepared by avoiding air bubbles between the gel, and membrane. A final crucial consideration for immunoblotting is effective optimisation of antibody concentration for specific samples, as too low and the signal will not be visible and too high could result in over-exposed (negative) bands on the blot, and high background signal. Altering membrane-washing times, the blocking reagent used and membrane exposure times can also dramatically affect signal-to-noise ratio, and therefore can also require optimisation.

X. laevis *egg quality*

A major critical parameter for nanotoxicity assessment in *X. laevis* embryos is the quantity and quality of egg production (and thus the zygotes generated from these), which has a major influence on the collection of reliable data. The *Xenopus* research community are aware that egg quality and production levels are variable, which is often attributed to differences between individual females. Therefore, experimental replication can be improved by acquiring eggs from consistently good producers. Acceptable methods for identification of individuals include tagging (with beads or microchips), tattooing, branding, monitoring of dorsal markings in pigmented frogs and perhaps more simply (if space is available), housing individuals in designated tanks. Implementing the following basic policies will increase the chances of quality egg harvests:

i) Comprehensive training of personnel preforming the procedures.

ii) Introducing a robust system for identifying individual animals.

iii) Ensuring a compulsory rest period of at least 4 months between ovulations (Green et al., 2007). This will allow females to be reused for several years provided they remain healthy.

iv) Daily monitoring of post-procedure females for up to two weeks in a separate recovery tank, to ensure there are no complications caused by ovarian hyper-stimulation (Green et al., 2007).

v) Detailed record keeping of all procedures conducted.

vi) Strict quarantine procedures for incoming animals into the aquarium.

There is also awareness in the community that *X. laevis* husbandry can also greatly influence egg quality. Seasonal changes, food, temperature, water quality and environmental enrichment are all factors that have been suggested to affect the quality and quantity of *X. leavis* eggs (Delpire et al., 2011; Godfrey and Sanders, 2004; Green, 2002; Hilken et al., 1995; Sigel, 1990; Wu and Gerhart, 1991). Although some of these effectors are difficult to control, they can be minimised by maintaining a 12 h light/12 h dark cycle, a constant temperature (21-23^{o}C), feeding once every 2-3 days, enriching the environment with functional items for the frogs (e.g. plastic plants, logs, dishes etc.) and careful monitoring of water quality.

NP exposure in* X. laevis *embryos

NP exposure time in *X. laevis* embryos is an important consideration for this protocol. Embryos must be exposed to NPs for a sufficient length of time in order for the key internal organs to be exposed to the nanomaterial being tested. It is also important to consider at what developmental stage the embryos are exposed to these nanomaterials. The described protocol can be adapted depending on the aims of the toxicity screen. For example, embryos can be exposed to NPs very early on during the developmental process, such as at NF stage 4 and

fixed at NF stage 38. Over this time, the embryos are exposed to NPs during key developmental processes such as gastrulation (NF stage 10) and neurulation (NF stage 15). The NF stage at which the NPs are applied will greatly affect exposure too. For example, between NF stages 38-45 the gills and mouth of the embryos are open, providing additional routes of exposure for NPs aside from the porous skin, as we have previously discussed (Webster et al., 2016). As highlighted in the protocol description, it is essential to confirm that the embryos have been exposed to the NPs being tested by the experimental end point, which we propose can be done using microscopy (see Support Protocol 5 and Alternate Protocol 1). This is of particular importance for nanomaterials that do not produce visible toxicity in the embryos.

Anticipated Results

NP physical characterization is crucial step in a toxicity evaluation of NP dispersions for both *in vitro* and *in vivo* experiments. Importantly, it is necessary to evaluate stability and size distribution of the NP dispersions in experimental conditions that mimic, as much as possible, the conditions similar to those used in the biological nanotoxicity assays (i.e. temperature, dispersion medium, NP dose, etc.). Stability of the NP dispersion in its dispersing medium does not guarantee that such NPs are equally stable in the media used in the biological study. Biological media are complex fluids containing biomolecules and salts that can strongly affect NP self-assembly in solution, in some cases also causing agglomeration and precipitation. It is known that NP cellular interaction and uptake are affected by NP physical properties and size, thus to interpret NP biological response it is necessary to know their features in the biological environment. DLS is the best technique to investigate the stability of the NP dispersions in different media over time at biologically relevant temperatures. It is important to note that this technique provides the hydrodynamic

size distribution of the NPs in the solution (highlighting possible aggregation effects), but it does not provide the exact size of the single NP. For this reason TEM experiments should be done to complement DLS investigation. TEM is an imaging technique that gives information on the morphology and size of the NPs, providing exactly the size of the NP units in the dispersion. This knowledge permits better interpretation of the DLS results. It is also important to underline that TEM sizes are not representative of the NP distribution in solution. In fact, the drying process necessary to measure the NPs, could promote agglomeration. Nevertheless, qualitative information can be extracted that can be related to NP dispersibility. In fact, if the images show single well-separated NPs on the grid, it is reasonable to assume that they are also well dispersed in the dispersion. In the same way if big NP agglomerates are visible in the grid, it suggests that NPs are also aggregated when dispersed in aqueous solutions.

Cytototoxicity assessment is an essential step in the described process of NP hazard assessment (Fig. i). As detailed above in basic protocol 2, the researcher should select cytotoxicity assessment methodologies that are compatible with their nanomaterials of choice (see critical parameters section; cell culture considerations for discussion). Ideally the selected methodologies should cover >1 cytotoxic assessment parameters (oxidative stress, cell death, cell viability and inflammatory response). Here we detail three protocols (basic protocol 2, support protocol 2 and support protocol 3) that combined, robustly assess cell viability (MTT and trypan blue exclusion assays) and cell death in response to NP treatment, providing percentage cell viability readings and an indication of apoptosis by immunoblotting. As detailed above, this data is then combined with results from the *X. laevis* phenotypic abnormality assay (basic protocol 3) to provide a hazard ranking score for NP safety assessment.

The *X. laevis* phenotypic abnormality assay (Basic Protocol 3) results in the percentage of embryos that did not survive NP exposure and the percentage that display phenotypic abnormalities relative to the total number of embryos tested, and therefore represents the percentage lethality and percentage abnormality, respectively. Expected abnormalities commonly include eye malformations, bent anterior-posterior axis, oedema, blistering, stunted growth and pigmentation loss (Table iii). We have previously described example results for a range of high-to-low toxicity-inducing nanomaterials (Webster et al., 2016). As discussed above, comparison between the *X. laevis* phenotypic abnormality data and the cytotoxicity results provides a hazard ranking score for NP safety, which can be used to determine whether or not further nanotoxicity assessment in mammalian systems is permissible or if further optimisation of NP design/synthesis is first needed to reduce toxicity of the developed nanoformulation (Fig. i).

Time Considerations

Basic Protocol 1: Preparation of the samples for DLS measurements is a quick procedure that generally involves the dilution of the NP stock dispersions in the different biological media. A DLS experiment is quite fast, it will take between 5-15 min depending on if the measurement is performed at fixed angle or at different angles (in the latter case it will be longer). The measurements should be repeated over the experimental time of the biological assay with closer repetitions in the first day. Overall, the experimental time depends on the sample numbers and duration of the biological experiments. Moreover, additional time should be considered for the analysis of DLS data for multi-angle measurements for which the operator needs to make some more analysis work after the experiments.

Support Protocol 1: Preparation of TEM samples on suitable grids requires at least overnight incubation to guarantee complete evaporation of the solvent. Generally, the grids will be analysed by a specialized technician, thus the experimental time is not predictable. The actual measurement takes approximately half an hour for sample (different areas of the grids need to be imaged). After that the operator will need to analyse the images with specific imaging softwares for extracting a size distribution of the NPs. The duration of this analysis depends on the quality of the images and the properties of the sample, if the NPs are well separated usually it is possible with most imaging softwares to automatically measure the size of all the NPs. While if the NPs formed agglomerates on the grid, size measurement of each single NP has to be done manually and this will take longer time.

Basic Protocol 2: Preparation of mammalian cell line stocks, validation and preparation of cells for experiments will take 2-3 weeks depending on how well the specific cells grow in culture. Seeding/growing cells will take 1 day and NP treatment takes 3 days. The MTT assay takes a further 5-6 h (depending on sample numbers) and the reading/generation of results ~1-2 h: ~3-4 weeks in total, depending on how well the cell lines grow.

Support Protocol 2: As stated above for basic protocol 2, cell line preparation, seeding and treating with NP will take ~2-3 weeks plus an additional 4 days. The trypan blue exclusion assay will take a further 30 min-2 h depending on how many samples are to be analysed. Likewise, cell counting will take 10 min-2 h depending on sample numbers and count methodology: ~3-4 weeks in total, depending on how well the cell lines grow.

Support Protocol 3: Sample preparation including treatment times and protein preparation will take ~4-5 days. SDS-PAGE and completion of immunoblotting will then take a further 0.5 and 3 days, respectively: ~7-8 days in total, depending on optimised conditions.

Basic Protocol 3: Depending on the requirements of the NF stage needed for specific experiments, *X. laevis* embryo exposure and incubation times can vary from a few h to several days. This is also influenced by the incubation temperature used (see points 1-4 of Basic Protocol 3 for discussion of time estimates). At the end of the incubation period, fixing the embryos can take 2-24 h depending on the temperature used. Washing, mounting, imaging and scoring the embryos will take a few hours depending on how many embryos need to be analysed. Finally, dehydration of embryos for long-term storage takes ~30-40 min: ~1 week in total.

Support Protocol 4: Priming of females can take up to 1 week and induction of ovulation, up to 14 h. Fertilisations and de-jellying will take 2.5 h: ~6-8 days in total.

Support Protocol 5: Preparation of imaging plates (1 h) and live, whole-mount fluorescent imaging of embryos ~1-3 h (depending on the number of embryos to analyse): 2-4 h in total.

Alternate Protocol 1: Anesthetising, fixing and dehydrating embryos takes 3.5 h in total. Embedding the embryos in resin takes 3 days, whilst sectioning, mounting and imaging could take up to 2-3 days (depending upon the number of samples to process): ~5.5-6.5 days in total.

Acknowledgments

This work was funded by the British Skin Foundation, The Royal Society and the Ministry of Higher Education and Scientific Research, Republic of Iraq. V.S. is supported by a CRUK programme grant awarded to the CRUK-Skin Tumour Laboratory, University of Dundee.

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Figure Legends

Figure i: Flow diagram of proposed nanotoxicity screening protocol. This figure is adapted from a previously published study from our group (Webster et al., 2016). Briefly, newly synthesised nanotherapeutics are firstly characterised in terms of their physicochemical characteristics in biologically relevant media (basic protocol 1). Once identified as stable by this protocol, NPs are further assessed through an integrated approach of cytotoxicity analysis and phenotypic abnormality screening in *X. laevis* embryos (basic protocol 2 and basic protocol 3, respectively). Here we provide detailed methodological descriptions of these three protocols as highlighted in the dotted box. Results from basic protocols 2 and 3 are then

combined to provide a score that can indicate whether or not further *in vivo* nanotoxicity assessment should be made using mammalian models.

Figure ii: Schematic drawing of a DLS apparatus with a multi-angle detector. The equipment is composed of a monochromatic laser in the visible range, optical lenses to focus the beam on the sample, attenuator of the incident light, detector (equipped with a motor to move it at different angles with respect to the incident beam), correlator and PC with a specific software for the analysis of the raw data. The attenuator modulates the incident light to an optimal value that depends on the features of the detector. The detected scattered light reaches the correlator that builds an auto-correlation function of the scattered intensity for each angle. The auto-correlation functions and the raw signals (kcounts/s) can be analyzed by the specific software provided by the supplier of the Instrument.

Figure iii: Suggested *X. laevis* NF stages for NP exposure. Schematic depicts *X. laevis* embryos at different developmental NF stages that have been selected for treatment to assess nanotoxicity (Webster et al., 2016). Embryo physiology images (Nieuwkoop and Faber, 1967) depicted above the line, with their associated NF staging description provided below the line. Images not to scale. The selected NF stages for NP exposure provide analysis of two critical teratogenic assessment stages; gastrulation (NF 4-NF 38) and neuralation (NF 15-NF 38), and at stages that can more accurately represent an adult system during organogenesis (NF 38-45).