Project: VIGO

EMPA
Materials Science & Technology

NM interference in the MTS assay

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Table of Content

1 Introduction

Nanomaterials (NM) have been shown to interfere in different *in vitro* assays (e.g. Belyanskaya, 2007; Casey, 2007; Guo, 2008; Monteiro-Riviere, 2006; Pulskamp, 2007; Wörle-Knirsch, 2006; for a review see also Kroll et al., 2009). To avoid false positive as well as false negative results it's thus important to elucidate possibilities of interference and to find ways to assess them experimentally. This SOP describes the theoretical considerations about potential interference reactions of NMs in a MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-cyrboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) cell viability assay and the experimental implementation.

2 Principle of the Method

The CellTiter 96® AQ_{ueous} One Solution (later on simply called MTS) contains the MTS reagent itself and an electron coupling reagent (phenazine ethosulfate;PES) in a stable solution. MTS is added directly to the cells. PES is membrane permeable, enters the cell and is reduced by mitochondrial enzymes (dehydrogenases involving NADPH or NADH), active only in viable cells. The reduced PES is then able to transform the MTS reagent to its formazan product. The resulting color is quantified by an absorbance measurement at 490 nm.

In terms of NM interference in this assay three possibilities have to be considered:

1. NMs stick to the cell culture plastic thereby generating an absorbance signal by themselves.
2. NMs are able to reduce MTS (either the MTS reagent directly or via the reduction of the electron coupling reagent (PES)).
3. The presence of NMs changes the absorbance value of the reduced MTS (formazan).

These considerations are addressed in cell free control experiments.

3 Applicability and Limitations

Values obtained in the cell free control experiments cannot be calculated against values from cellular measurements. They serve as qualitative estimations of NM only reactions that do not involve cellular contribution.

4 Related Documents

Table 1: Documents needed to proceed according to this SOP and additional NM-related interference control protocols.

Document ID	Document Title
M_NM suspension_metal oxides	*Suspending and diluting Nanomaterials – Metal oxides and NM purchased as monodisperse suspensions*
M_NM suspension_ carbon based	*Suspending and diluting Nanomaterials – Carbon based nanomaterials*

5 Equipment and Reagents

5.1 Equipment

- Absorbance reader for multi-well plates (to measure optical density (OD) at a wavelength of λ=490 nm)
- Conical tubes (15 ml and 50 ml; polypropylene or polystyrene; e.g. from Falcon)
- Flat bottom 96-well cell culture plates
- Microreaction tubes (1.5 ml; e.g. from Eppendorf)
- Multichannel pipette (with at least 8 positions; volume range per pipetting step at least from 50 µl to 200 µl)
- Vortex®

5.2 Reagents

To dilute carbon based NM:

- 10x concentrated RPMI-1640
- Sodium bicarbonate solution, 7.5% ($NaHCO_3$) *[CAS-number: 144-55-8]*

Buffers, solvents and detection dye itself:

- CellTiter96® AQ_{ueous} One Solution *[Promega; Cat. No. G3580-G3582]*
- Sodium sulfite (Na_2SO_3) *[CAS number: 757-83-7]*
- Roswell Park Memorial Institute Medium (RPMI-1640) WITHOUT phenol red
- Pluronic F-127 *[CAS number: 9003-11-6]*

5.3 Reagent Preparation

5.3.1 Sodium sulfite (Na_2SO_3)

Stock:

- 100 mM in ddH_2O: 1.26 g/100 ml

5.3.2 Pluronic F-127

Stock:

- 160 ppm in ddH_2O: 160 µg/ml (=16 mg/100 ml)

6 Procedure

6.1 Dilution of nanomaterials

For this SOP we distinguish two types of nanomaterials (NM) according to their solvent, suspension properties and highest concentrations used in the assay. See also respective related documents (3).

(1) Metal oxide NM, Polystyrene beads and all NM delivered as monodisperse suspensions by the supplier: solvent either determined by the supplier or ddH_2O; sub-diluted in ddH_2O; highest concentration in assay 100 µg/ml
(2) Carbon based NM: suspended and sub-diluted in 160 ppm Pluronic F-127; highest concentration in assay 80 µg/ml

Volumes given in the following dilution schemes are enough for one 96-well plate.

Note: "Mixing" in the context of diluting NMs means, the solvent containing tube is put on a continuously shaking Vortex® and the previous sub-dilution (or stock suspension, respectively) is put dropwise into the shaking solvent. The resulting suspension stays on the Vortex® for additional 3 seconds before proceeding with the next sub-dilution.

(1) Metal oxide NM:

Prepare serial sub-dilutions of the stock suspension (1 mg/ml) in ddH_2O:

- Label ten conical tubes (15 ml total volume) with 1 to 10 (relates to steps 1-10 below).
- Add 1.8 ml ddH_2O to tube 1.
- Add 1.5 ml ddH_2O to tubes 2 to 10.

1. 1.2 ml NM stock suspension (1 mg/ml) are mixed with 1.8 ml ddH_2O → 400 µg/ml (1)
2. 1.5 ml of 400 µg/ml (1) are mixed with 1.5 ml ddH_2O → 200 µg/ml (2)
3. 1.5 ml of 200 µg/ml (2) are mixed with 1.5 ml ddH_2O → 100 µg/ml (3)
4. 1.5 ml of 100 µg/ml (3) are mixed with 1.5 ml ddH_2O → 50 µg/ml (4)
5. 1.5 ml of 50 µg/ml (4) are mixed with 1.5 ml ddH_2O → 25 µg/ml (5)
6. 1.5 ml of 25 µg/ml (5) are mixed with 1.5 ml ddH_2O → 12.5 µg/ml (6)
7. 1.5 ml of 12.5 µg/ml (6) are mixed with 1.5 ml ddH_2O → 6.25 µg/ml (7)
8. 1.5 ml of 6.25 µg/ml (7) are mixed with 1.5 ml ddH_2O → 3.13 µg/ml (8)
9. 1.5 ml of 3.13 µg/ml (8) are mixed with 1.5 ml ddH_2O → 1.56 µg/ml (9)
10. 1.5 ml ddH_2O → solvent control (10)

Final dilutions are prepared directly in the 96-well plate during assay performance as shown in Figure 2.

(2) Carbon based NM:

Prepare serial sub-dilutions of the stock suspension (500 µg/ml) in 160 ppm Pluronic F-127:

- Label ten conical tubes (15 ml total volume) with 1 to 10 (relates to steps 1-10 below).
- Add 1.08 ml 160 ppm Pluronic F-127 to tube 1.
- Add 1.5 ml 160 ppm Pluronic F-127 to tubes 2 to 10.

1. 1.92 ml NM stock suspension (500 µg/ml) are mixed with 1.08 ml Pluronic F-127 → 320 µg/ml (1)
2. 1.5 ml of 320 µg/ml (1) are mixed with 1.5 ml Pluronic F-127 → 160 µg/ml (2)
3. 1.5 ml of 160 µg/ml (2) are mixed with 1.5 ml Pluronic F-127 → 80 µg/ml (3)

4. 1.5 ml of 80 µg/ml (3) are mixed with 1.5 ml Pluronic F-127 → 40 µg/ml (4)
5. 1.5 ml of 40 µg/ml (4) are mixed with 1.5 ml Pluronic F-127 → 20 µg/ml (5)
6. 1.5 ml of 20 µg/ml (5) are mixed with 1.5 ml Pluronic F-127 → 10 µg/ml (6)
7. 1.5 ml of 10 µg/ml (6) are mixed with 1.5 ml Pluronic F-127 → 5 µg/ml (7)
8. 1.5 ml of 5 µg/ml (7) are mixed with 1.5 ml Pluronic F-127 → 2.5 µg/ml (8)
9. 1.5 ml of 2.5 µg/ml (8) are mixed with 1.5 ml Pluronic F-127 → 1.25 µg/ml (9)
10. 1.5 ml 160 ppm Pluronic F-127 → solvent control (10)

Final dilutions are prepared directly in the 96-well plate during assay performance as shown in Figure 2.

6.2 Reduction of MTS to formazan

Volumes are given for one 96-well plate as shown in Figure 1.

- 5 ml RPMI without phenol red are mixed with 1 ml MTS and 50 µl 100 mM Na_2SO_3.
- Mix on the vortex.
  Note: The yellow MTS turns almost immediately into its brownish formazan product.

6.3 MTS working solution

- 5 ml RPMI without phenol red are mixed with 1 ml MTS.

6.4 Application into 96-well plate

Make sure to have sub-dilutions of NMs ready.

Note: All NM dilutions have to be vortexed directly before application.

- Distribute 100 µl per well of RPMI without phenol red (white wells), MTS working solution (yellow wells) and formazan (in RPMI without phenol red; brown wells) into a 96-well plate as shown in Figure 1.
- Black wells remain empty.

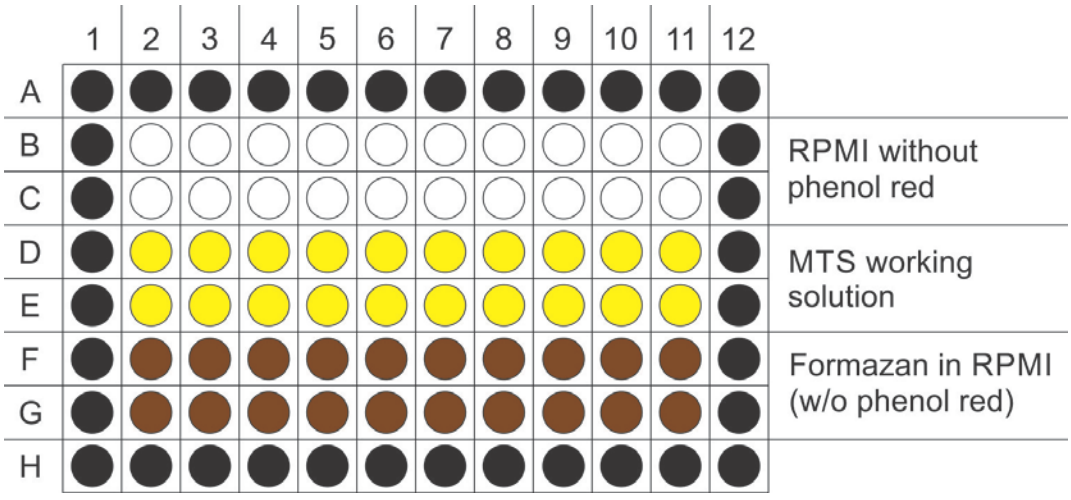

Figure 1: Distribution of RPMI without phenol red, MTS working solution and Formazan in RPMI (w/o phenol red) into a 96-well plate.

- Add 100 µl of the appropriate NM sub-dilution per well as shown in Figure 2.
  Note: Due to the 1:2 dilution of the NM sub-dilutions in RPMI, MTS or Formazan, respectively, final concentrations are halved.
- Outermost wells (black in Figure 2) remain empty.

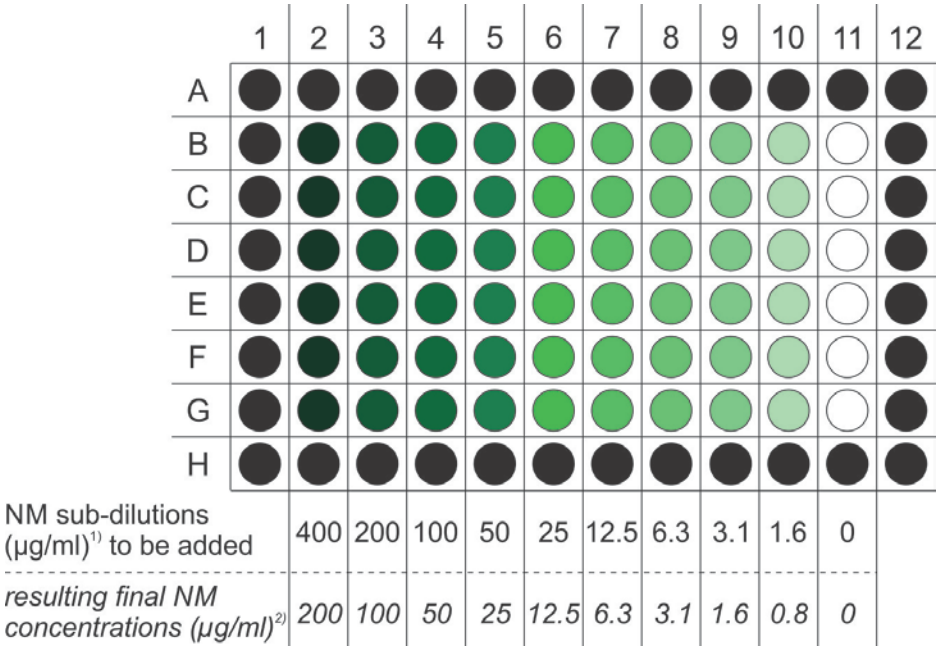

Figure 2: Application of NM.
Add 100 µl of the respective NM sub-dilution per well of the 96-well plate. Outermost wells remain empty. Incubate for 60 min in a humidified incubator at 37°C and 5% CO_2.
[1)] NM concentrations given here refer to metal oxide NM. Carbon based NM concentrations are detailed in the text.
[2)] Due to the 1:2 dilution of NM sub-dilutions in RPMI, MTS or Formazan, respectively, final concentrations are halved.

- Incubate the 96-well plate for 60 min in a humidified incubator at 37°C and 5% CO_2.
- Measure the absorbance at 490 nm in a plate reader.

6.5 Data evaluation

Calculate the mean of the six technical replicates of each concentration. These mean absorbance values are plotted in a bar chart or as a dot plot to compare treated and untreated samples directly. Furthermore, the effect can be expressed in percent of the untreated/solvent treated control.

7 Quality Control, Quality Assurance, Acceptance Criteria

8 Health and Safety Warnings, Cautions and Waste Treatment

9 Abbreviations

ddH_2O	double-distilled water
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-cyrboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NM	Nanomaterial
OD	Optical density
PES	Phenazine ethosulfate
ppm	parts per million
RPMI	Roswell Park Memorial Institute medium

10 References

Belyanskaya L, Manser P, Spohn P, Bruinink A, Wick P; 2007: The reliability and limits of the MTT reduction assay for carbon nanotubes-cell interaction. *Carbon* 45: 2643-2648

Casey A, Herzog E, Davoren M, Lyng FM, Byrne HJ, Chambers G; 2007: Spectroscopic analysis confirms the interactions between single walled carbon nanotubes and various dyes commonly used to assess cytotoxicity. *Carbon* 45: 1425-1432

Guo L, von dem Bussche A, Buechner M, Yan A, Kane AB, Hurt RH; 2008: Adsorption of essential micronutrients by carbon nanotubes and the implications for nanotoxicity testing. *Small* 4: 721-727

Kroll A, Pillukat MH, Hahn D, Schnekenburger J; 2009: Current *in vitro* methods in nanoparticle risk assessment: Limitations and challenges

Monteiro-Riviere NA, Inman AO; 2006: Challenges for assessing carbon nanomaterial toxicity to the skin. *Carbon*44: 1070-1078

Pulskamp K, Diabate S, Krug HF; 2007: Carbon nanotubes show no sign of acute toxicity but induce intracellular reactive oxygen species in dependence on contaminants. *Toxicol. Lett.* 168: 58-74

Wörle-Knirsch JM, Pulskamp K, Krug HF; 2006: Oops they did it again! Carbon nanotubes hoax scientists in viability assays. *Nano Lett.* 6: 1261-1268