CYTOTOXICITY INDUCED BY NANOPARTICLES – STANDARTISATION OF METHODS WITHIN QUALITYNANO FP7 NETWORK

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Abstract

Nanomaterials (NMs) have become crucial for many applications promising significant improvement in various aspects of quality of life. Indisputable benefits of NMs, however, might be discredited by concerns about potential adverse health effects arising from unique properties related to the nanosize. Safety assessment of NMs is complicated by the fact that conventional methods are insufficient for evaluating their toxicity. Although new methods adapted for NMs are being extensively introduced by toxicologists, the lack of standardization causes inconsistency in existing toxicity data making it impossible to draw general conclusions about NM toxicity.

QualityNano is a current European FP7 project aimed at developing and implementing best practice and quality in nanosafety assessment. As one of the participating laboratories, we evaluated cytotoxic potential of two reference NMs (non-cytotoxic 40nm carboxy-functionalized and cytotoxic 50nm amino-functionalized polystyrene nanoparticles) in lung adenocarcinoma A549 cell line using QualityNano Standard Operation Procedure for MTS Cell Viability Assay. Results of two performers showed good intra-laboratory concordance and obtained data were provided for inter-laboratory variation assessment which is currently being in progress within QualityNano Network.

The same protocol was used to evaluate cytotoxicity of the reference NM sample in immortalized bronchial epithelial BEAS-2B cell line. As expected, BEAS-2B cells were more susceptible than cancerous A549 cells.

Finally, two other methods were employed to investigate concordance among various cytotoxicity assays. Both metabolic activity-based WST-1 assay and LDH membrane integrity assay have been proven to be relevant for studying NM cytotoxicity.

Keywords: Nanomaterials, Cytotoxicity, Standardization, QualityNano

1. INTRODUCTION

Nanomaterials (NMs) and nanotechnologies are considered to be one of the future key technologies, promising breakthroughs in vital fields, such as healthcare, energy, environment, or manufacturing [1]. However, as it is the case of any new technology, early phase of NM applications and implementations is naturally accompanied by uncertainties and concerns about their safety. Unregulated use of new materials might lead to serious damage to human health and environment. On the other hand, if not managed appropriately, the potential risks of NM exposure can reduce public confidence and give rise to rejection of nanotechnology as a whole.

Safety assessment of NMs is complicated by the fact that conventional methods are insufficient to adequately evaluate their potential toxicity [2]. To avoid misleading conclusions, modified or novel approaches are required to be designated and validated to gather reliable information base on toxicity

of NMs. Moreover, diversity and dynamic changes of NM physicochemical properties further hamper obtaining reliable and consistent toxicity data. It has been shown that even minor differences in testing or sample preparation procedures can change NM properties, e.g. particle size [3] or protein corona composition [4], and subsequently modify their biological effects. Thus standardized and generally acceptable protocols ensuring reproducible performance of experiments are necessary to produce comparable toxicity data.

The issue of quality in nanosafety research is thoroughly addressed within FP7 project QualityNano. QualityNano integrates 31 top European analytical & experimental facilities in nanotechnology, medicine and natural sciences with the goal of developing and implementing best practice and quality in all aspects of nanosafety assessment [5]. As a participating laboratory, we are involved in inter-laboratory trial to assess variation in NM cytotoxicity testing using reference nanoparticles (NPs) and Standard Operation Procedure generated in scope of Network Activity 2 - Nanomaterials Hub & Round-Robin Assessment.

QualityNano protocol originally designed for cytotoxicity evaluation of reference NPs using MTS assay in A549 cells was adapted to examine other common *in* vitro cytotoxicity methods, WST-1 and LDH assay. In addition, susceptibility of two most widely used human lung cell lines, cancerous A549 and normal BEAS-2B, towards the reference cytotoxic NPs were investigated in three *in vitro* systems (MTS assay, WST-1 assay, LDH assay).

2. METHODS

2.1 Nanomaterial source and storage

Non-cytotoxic 40nm carboxy-functionalized (PS-COOH NP) and cytotoxic 50nm amino-functionalized polystyrene nanoparticles (PS-NH_2 NP) were provided within the QualityNano network for inter-laboratory variation assessment of NP cytotoxicity testing. NPs were prepared, characterized and distributed to participating laboratories by University College Dublin (UCD). Participating laboratories obtained stock solutions of 10 mg/ml NPs in H_2O. Stock solutions were stored at 4 ºC and freshly diluted prior to testing.

2.2 Cell culturing and seeding

A549 (Human lung epithelial carcinoma) cells were grown according to the QualityNano Standard Operating Procedure in MEM with L-Glutamax (Invitrogen Life Technologies) supplemented with 10% non-heat inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2.

BEAS-2B (human bronchial epithelial) cells were cultured in F12/DMEM media (1:1) supplemented with 10% non-heat inactivated FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 5 % CO_2.

For cytotoxicity experiments, cells were seeded in 96-well microplates at density of 5×10^4 cells/ml in 100 µl of complete cell culture medium (cCCM) (i.e. 5×10^3 cells/well). Cells were incubated for 24 hours prior to exposure.

2.3 Cell exposure

Working solutions of NPs were prepared in technical triplicates immediately before cell exposure by diluting the stock solution (10 mg/ml NPs in H_2O) in cell culture medium containing 10 % or 1 % FBS to yield a concentration of 100 µg/ml. 10 % FBS was used in plates for MTS and WST-1 assay, 1 % FBS was applied for LDH release measurement, since LDH present in serum enhances background absorbance. The working NP solution was then used to prepare dosing plate containing serial dilutions of NPs in the range of 1-100 µg/ml. Both NP stock and working suspensions were vortexed for 30s at full speed before using. Staurosporine in triplicates in five different concentrations ranged from 62.5 to 1000 nM was used as

a positive control. Six wells containing cells and cCCM without treatment were set up as a negative control. To determine background absorbance, six wells containing only medium without cells were included in the microplate. NP interference with the assays was examined in six wells containing only media and passing treatment without cells added.

After removing the media from the cell plate, the content of the dosing plate was transferred to the appropriate wells in plate containing cells and incubated for 24 hours at 37°C in a 5% CO_2 atmosphere.

2.4 MTS Assay

The assay was performed using CellTiter 96® AQueous One Solution Reagent (Promega) according to the QualityNano protocol. Briefly, after 24-hour exposure treatments were removed using multichannel pippetor and 120 µL of the MTS Reagent in cCCM was added into each well of the plate. After incubation at 37 °C for 1 hour in a humidified 5 % CO_2 atmosphere, light absorbance was measured at wavelength of 490 nm by SpectraMax® M5 Plate Reader (Molecular Device, USA). Cell viability was expressed as a percentage of control after subtracting background absorbance values.

2.5 WST-1 Assay

Commercially available kit Cell Proliferation Reagent WST-1 (Roche Diagnostics) was used as another mitochondrial activity-based assay to evaluate PS-NH_2 NP cytotoxicity. According to the manufacturer's instructions, 10 µl of the WST-1 reagent was added into each well at the end of 24-hour incubation period and plates were further incubated for 1 hour at 37 °C in a 5 % CO_2 atmosphere. After centrifugation at 4000 RPM for 20 min, 50 µl of the supernatants were transferred from each well into a new 96-well plate to remove NPs interfering with the absorbance reading. The absorbance at 450 nm was measured using SpectraMax® M5 Plate Reader (Molecular Device, USA) both before and after centrifugation. Cell viability was expressed as a percentage of control after subtracting background absorbance values.

2.6 LDH Assay

Cytotoxicity Detection Kit (LDH) (Roche Diagnostics) was used to evaluate cytotoxic potential of PS-NH_2 NPs by measuring the degree of cellular membrane damage. After 24-hour exposure, 50 µl of cell culture supernatant from each well was transferred to a corresponding well on a new plate. After removing the rest of the culture media, cells were lysed using 1% Triton-X100 in 1 % CCM at 37 °C for 30 min and 50 µl of the lysates were transferred to a new plate. 50 µL of assay Reaction Mixture was added to both the supernatants and the cell lysates and incubated at room temperature. After 15 minutes of incubation, 25 µL of Stop Solution was added to each well to terminate the reaction. The absorbance at 490 nm was measured using SpectraMax® M5 Plate Reader (Molecular Device, USA). Cell viability was calculated as the ratio of LDH release (supernatants) to the maximum LDH release (supernatants + lysates). The control was set to 100% viability and the results were expressed as percentage of cell viability.

2.7 Data analysis

All results were expressed as mean ± SD of at least two independent experiments performed in triplicates.

3. RESULTS AND DISCUSSION

As one of QualityNano participating laboratories, we obtained samples of two reference NMs 1) non-cytotoxic 40nm carboxy-functionalized polystyrene nanoparticles (PS-COOH NP) and 2) cytotoxic 50nm amino-functionalized polystyrene nanoparticles (PS-NH_2 NP). As far as recommendations on NP storage and preparation are followed, stability and dispersion of the NPs in suspension is guaranteed by the provider (UCD). Characterization of the polystyrene NPs was carried out by UCD. Basic characteristics of the tested particles are summarized in **Table 1**.

Table 1 Characterization of the reference NPs

Particle name	*Chemical composition*	*Functionalization*	*Particle size*	*Biological effects*
PS-NH2 NP	Polystyrene	NH_2	50 nm	Cytotoxic
PS-COOH NP	Polystyrene	COOH	40 nm	Non-cytotoxic

3.1 Intra-laboratory concordance of cytotoxicity testing of standard polystyrene NPs using QualityNano protocol for MTS assay

Cytotoxic potential of the reference polystyrene NPs in A549 cells were evaluated using MTS assay according to the QualityNano Standard Operation Procedure. As expected, viability of cells exposed to cytotoxic NPs decreased in dose-dependent manner, whereas viability of cells exposed to non-cytotoxic NPs were not affected (see Fig. 1). Results produced by two performers showed good intra-laboratory concordance. Obtained data were provided for inter-laboratory variation assessment which is currently being in progress within QualityNano Network.

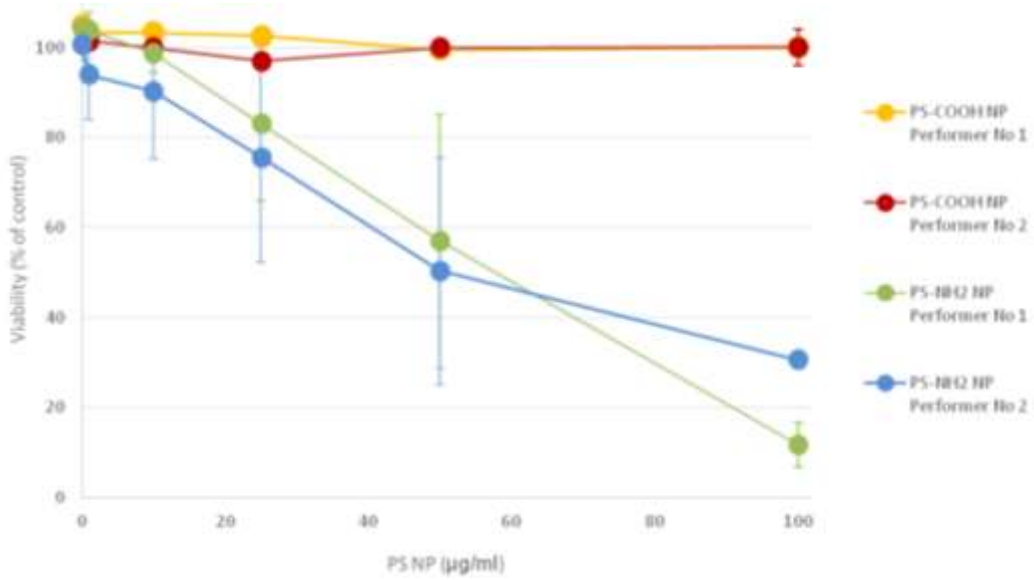

Fig. 1 Intra-laboratory concordance of cytotoxicity assessment of reference polystyrene NPs using QualityNano Standard Operation Procedure. A549 cells were treated with different concentrations of cytotoxic (PS-NH_2 NP) and non-cytotoxic (PS-COOH NP) polystyrene nanoparticles for 24 hours. Data are shown as means ± SD from two independent experiments (each sample in triplicate) for each of two performers.

3.2 Cytotoxic effects of reference polystyrene NPs using MTS assay, WST-1 assay and LDH assay in A549 cell line

Following the QualityNano protocols, concordance among three commonly used *in vitro* cytotoxicity assays was investigated. As in the case of MTS assay, both WST-1 assay and LDH membrane integrity assay were able to detect dose-dependent cytotoxicity of the reference PS-NH_2 NPs (Fig. 2).

Interestingly, enhanced absorbance values were observed with increasing PS-NH_2 NPs concentration in WST-1 assay (absorbance measured at 450 nm) in control wells containing only medium and NPs. NP light absorbance in wells with the highest NP concentration (100 µg/ml) resulted in approx. two-fold increase in cell viability, providing false negative cytotoxicity results (data not shown). After subtracting background absorbance of media containing appropriate concentrations of NP, relevant cytotoxicity data were obtained.

The same viability rates were achieved when NPs were removed by centrifugation and light absorbance was measured in supernatants only.

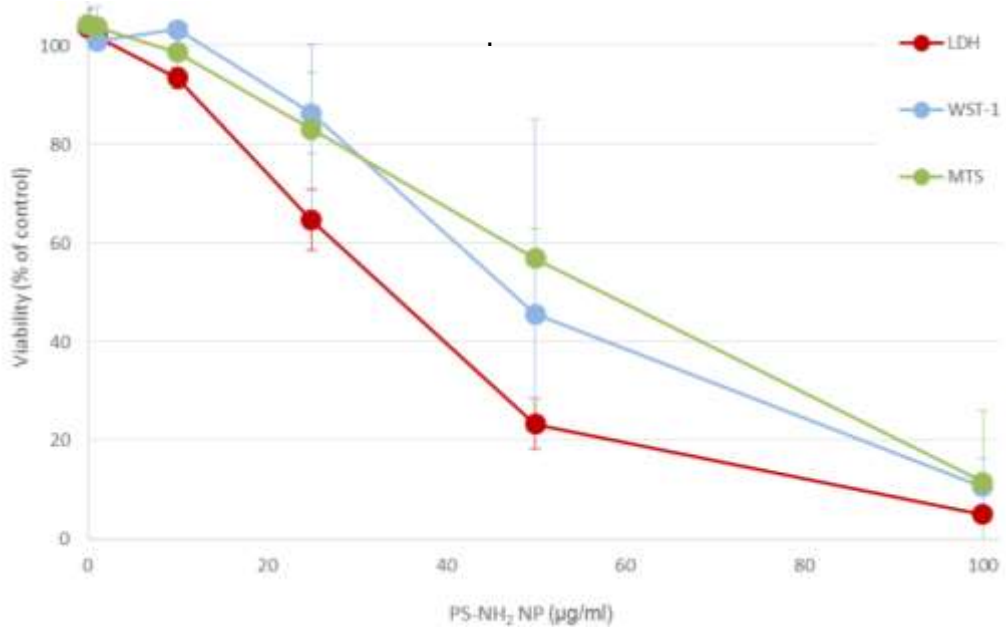

Fig. 2 Cytotoxicity of reference polystyrene NPs in three in vitro cytotoxicity assays. A549 cells were treated with different concentrations of cytotoxic amino-functionalized polystyrene NP. After 24-hour exposure, MTS, WST-1 and LDH assays were employed to evaluate cytotoxic potential of the NP. Data are expressed as means ± SD from two independent experiments performed in triplicates.

3.3 Cytotoxicity of reference polystyrene NPs in BEAS-2B cell line

In addition to adenocarcinoma A549 cells, immortalized BEAS-2B cell line was tested to compare susceptibility of the two most widely used human lung cell lines, cancerous A549 and virus-transformed BEAS-2B (Fig. 3).

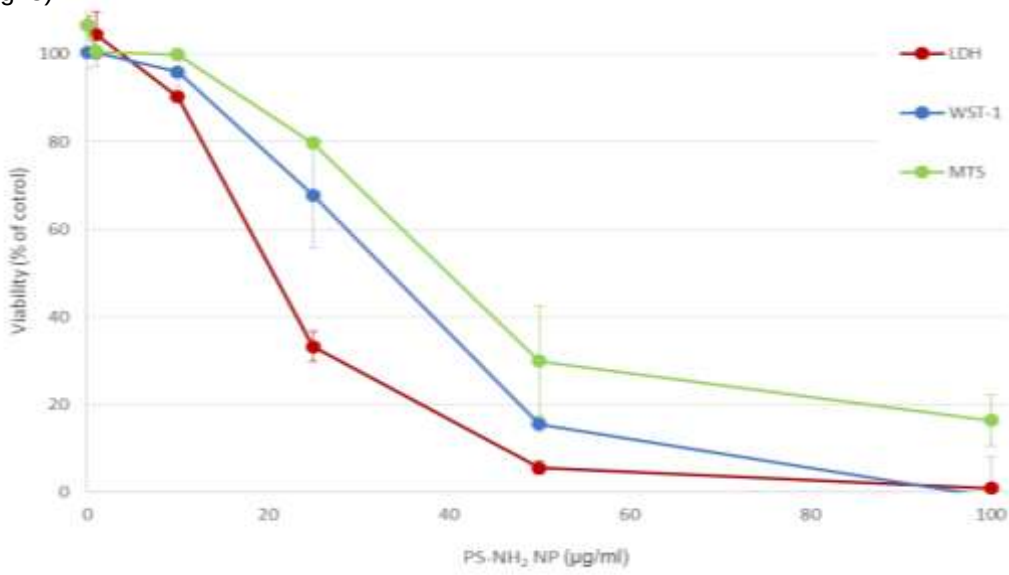

Fig. 3 Cytotoxicity of reference polystyrene NPs in BEAS-2B cell line. BEAS-2B cells were treated with different concentrations of cytotoxic amino-functionalized polystyrene NPs. After 24-hour exposure, MTS, WST-1 and LDH assays were employed to evaluate cytotoxic potential of the NP. Data are expressed as means ± SD from two independent experiments performed in triplicates.

The values of LC50 (i.e. NP concentration lethal to 50% of exposed cells) summarized in Table 2 indicate slightly higher susceptibility of BEAS-2B cells in comparison to A549 cell line in all performed cytotoxicity assays. This observation points out the possibility of hazard underestimation when using resistant cancerous cell lines.

Table 2 LC50 values of cytotoxic NPs in BEAS-2B and A549 cells. **LC50 v**alues were derived from viability curves in Fig. 2 and Fig. 3.

	MTS	***WST-1***	***LDH***
A549	60 µg/ml	50 µg/ml	33 µg/ml
BEAS-2B	40 µg/ml	33 µg/ml	20 µg/ml

4. CONCLUSIONS

Wide spectrum of NMs being tested under variable conditions and using non-standardized testing protocols lead to production of data which cannot be used to draw solid conclusion on factors underlying NM toxicity [6]. To achieve reliability and reproducibility of nanotoxicological data, standardized protocols for NP characterization, sample preparation and testing should be implemented in laboratories worlwide.

Herein, we evaluated cytotoxic potential of two reference NMs (non-cytotoxic 40nm carboxy-functionalized and cytotoxic 50nm amino-functionalized polystyrene NPs) in lung adenocarcinoma A549 cell line using QualityNano Standard Operation Procedure for MTS Cell Viability Assay. Results of two performers showed good intra-laboratory concordance and obtained data were provided for inter-laboratory variation assessment which is currently being in progress within QualityNano Network.

Using the same protocol, metabolic activity-based WST-1 assay and LDH membrane integrity assay have been proven to be relevant for studying NM cytotoxicity. Finally, immortalized bronchial epithelial BEAS-2B has been shown to be more susceptible to reference NM cytotoxic effects in all performed assays (MTS, WST-1, LDH assay) than so far the most widely used lung A549 cells.

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