Chapter 19

Cytotoxic Assessment of Carbon Nanotube Interaction with Cell Cultures

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Abstract

The field of nanotoxicology recently has emerged out of the need to systematically study the biocompatibility and potential adverse effects of novel nanomaterials. Carbon nanotubes (CNT) are one of the most interesting types of nanomaterials, and recently, their use in applications has dramatically increased. Their potential adverse impact on human health and the environment, however, have caused them to be viewed with apprehension in certain cases so further studies into their toxicology are justified. Current methodologies using cell culture (in vitro) models are unreliable and are not yet able to offer conclusive results about the toxicity profile of CNT. The need for reliable and rapid toxicity assays that will allow high throughput screening of nanotube materials is a prerequisite for the valid assessment of CNT toxicity. The assay described here was developed based on the pitfalls and drawbacks of traditionally used cytotoxicity assays. A methodological description of the main problems associated with the MTT and the LDH assays is offered to illustrate the advantages of this novel assay for the study and determination of the cytotoxic profile of CNT. Most importantly, a thorough account of this novel assay which is considered to be rapid, reliable, and suitable for broad-spectrum cytotoxicity screening of different types of CNT is described.

Key words: Nanotechnology, Nanotoxicology, MTT, LDH, Fluorescence, Cell death, Apoptosis

1. Introduction

Carbon nanotubes (CNT), novel cylindrical nanostructures, have already exceeded many expectations in terms of widespread usage and large-scale manufacturing due to their extraordinary properties which include high electrical and thermal conductivity and robust mechanical properties (1). CNT also can be utilized as components in a variety of biomedical applications ranging from probes in biosensing strategies to drug delivery vectors in therapeutic schemes (1–6). However, before such applications are used

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in a clinical setting systematic toxicological assessment of CNT is needed and warranted.

Despite the increased usage of CNT, general conclusions about their in vitro cytotoxicity have proven difficult because of the wide variety of available CNT materials (e.g., in terms of their manufacturing, colloidal dispersion, and chemical purity) and the variability in the assays used to determine their toxicity. The interaction between CNT and the molecules used in traditional and well-established toxicity assays is one key factor attributing to this variability (7–12). Most traditional assays are based on colorimetry and fluorescence, and the CNT strongly interact with the chromophore molecules used. CNT also intrinsically interact through supramolecular stacking and assembly with species, such as macromolecules (e.g., polymers, proteins, and nucleic acids) and small molecules (e.g., doxorubicin).

Worle-Knirsch et al. (7) have previously indicated that the MTT assay is unreliable to use with CNT due to the commonly occurring false-positive results caused by the strong interaction between the CNT and the insoluble formazan crystals. They suggested using alternative cytotoxicity assays (such as LDH and flow cytometry with Annexin V/PI staining) and other tetrazolium-based assays (WST-1, INT, XTT). Casey et al. confirmed through spectroscopic analysis that single-walled carbon nanotubes (SWNT) interact with the dyes (Coomassie Blue, Alamar Blue, Neutral Red, MTT, and WST-1) used in cytotoxicity assays and concluded that most are not suitable for the quantitative toxicity assessment of CNT (8). In an attempt to propose a reliable cytotoxicity assay that would not rely on light absorbance, the same group described a novel approach using the clonogenic assay (13). Although reliable this assay is too time-consuming to allow for rapid screening. No other report has used the clonogenic assay since this work was published, and despite the reported inaccuracies most in vitro toxicity studies are still carried out using colorimetry-based methodologies. More recently, Monteiro-Riviere et al. (11) studied the reliability of a range of widely used viability and cytotoxicity assays with many types of nanoparticles including SWNT. They also found that SWNT interfered to varying degrees with the results of most established toxicity assays. The need for reliable toxicity assays that would allow rapid screening of nanomaterials has now become a serious obstacle toward safety validation of the myriad types of CNT as well as other types of nanoparticles.

Here, we propose a modified version of one of the most widely used and established cytotoxicity assays, the lactate dehydrogenase (LDH) assay, that circumvents all interactions between CNT and the fluorophore molecules leading to a reliable and technically straightforward methodological solution. A comparison with other established assays like the MTT and the original

LDH assay is also provided to illustrate the pitfalls and problems with those methodologies compared to the proposed *modified LDH* (mLDH) method that offers a trustworthy and reproducible determination of cellular toxicity following their interaction with CNT.

2. Materials

2.1. CNT Preparation

1. Pristine multiwalled carbon nanotubes (MWNT) (Nanocyl, Belgium) (see Note 1).
2. Pluronic F127 copolymer (Sigma, UK).
3. Sterile deionized water.
4. Glass vials.
5. Water bath.
6. Bath sonicator (Ultrasonic cleaner, VWR).

2.2. Cell Culture

1. Adherent cells, such as the lung epithelial cell line A549 (CCL-185, ATCC, UK), or others.
2. 0.05% Trypsin with 0.53 mM ethylenediaminetetraacetic acid (EDTA) tetrasodium salt (Gibco, Invitrogen, UK).
3. Culture media appropriate for the cell line being studied. F12 Ham media supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin, and 50 μg/mL streptomycin (all from Gibco, Invitrogen, UK) was used with A549 cells.
4. 96-Well flat bottom plate (Corning Costar Corporation®, USA).
5. 10% v/v Dimethyl sulfoxide (DMSO) (>99.7%, Hybri-Max™, sterile filtered, hybridoma tested) in complete cell culture medium.
6. 1, 5, and 25 mL serological pipettes.
7. Incubate at 37°C with 5% CO_2.
8. Trypan blue dye exclusion assay kit.

2.3. In Vitro Cytotoxicity Assays

2.3.1. MTT Assay

1. MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) powder (Sigma, UK). Stored at 4°C before reconstitution.
2. Sterile phosphate-buffered saline (PBS) (1×) (Gibco, Invitrogen, UK).
3. Sterile filter (0.22 μm).
4. DMSO, 100%.
5. Plate reader.

2.3.2. LDH Assay (Original and Modified)

1. LDH kit: CytoTox 96 non-radioactive cytotoxicity assay (Promega, UK) containing substrate mix (five vials), assay buffer (60 mL), LDH positive control (25 μL), lysis solution (3 mL) (see Note 2), and stop solution (65 mL). Store substrate mix and assay buffer at −20°C, protected from light until use. Store LDH positive control, lysis solution, and stop solution at 4°C.
2. 96-Well flat bottom plate (Corning Costar Corporation®, USA).
3. Phenol-free media (e.g., RPMI media) (Gibco, Invitrogen, UK).
4. 9% v/v Triton X-100.
5. Plate reader.

3. Methods

3.1. Preparation of CNT Dispersions

1. In a glass vial, hydrate the Pluronic F127 to a final concentration of 1% w/v with sterile deionized water.
2. Place the vial in a water bath (37°C) for 30–45 min or until the Pluronic F127 flocculates disappear.
3. Disperse 1 mg/mL pristine MWNT powder in 1% (10 mg/mL) Pluronic F127 by bath sonication for 30–45 min (see Note 3).
4. Store the stock MWNT:F127 dispersion at 4°C until further use. When ready to use, sonicate for 15 min.
5. Use the 1% F127 stock solution for the controls in the toxicological assessments. Store at 4°C until further use.

3.2. Cell Culture

The following protocol describes the incubation of the MWNT:F127 dispersions and control samples with A549 lung epithelial cell lines. If desired, the cells can be treated with various inhibitors and the incubation times of the samples with the cells and CNT concentration in the samples can be varied. DMSO is used as a positive control for cytotoxicity.

1. Passage A549 cells when they reach 70–80% confluency to maintain exponential growth. Use for a maximum of ten passages.
2. To trypsinize the monolayer, rinse with 1× PBS then incubate with trypsin–EDTA at 37°C for 5 min. Detach the cells by vigorous up and down pipetting.
3. Centrifuge the cells at 240 × *g* for 5 min at 4°C and resuspend in complete media.
4. Count cells and determine cell viability by Trypan blue dye exclusion assay.
5. Seed 10,000 cells per well (150 μL/well) in a 96-well plate and incubate for 24 h at 37°C in a humidified atmosphere (5% CO_2) incubator.

6. Dilute the stock MWNT:F127 dispersion or stock 1% F127 in complete cell culture medium to reach the desired concentrations (see Note 4).
7. Sonicate the diluted MWNT:F127 dispersion in media for 2 min prior to its addition to cells.
8. Incubate the cells with the CNT dispersions or control samples (1% F127 or 10% DMSO) for 24 h at 37°C in a humidified atmosphere (5% CO_2) incubator. Also, leave some cells untreated as controls.
9. Proceed with the cytotoxicity assessments (see Subheading 3.3).

3.3. In Vitro Cytotoxicity Assays

3.3.1. MTT Assay

The colorimetric MTT assay is used to measure cell viability (14). The yellow tetrazolium salt (MTT) is reduced by mitochondrial reductase in living and metabolically active cells to purple, water-insoluble formazan crystals, which can then be dispersed using DMSO or other detergents. A decrease in absorbance at 570 nm compared to untreated control cells is then a measure of the cell viability or the amount of apoptosis or necrosis that has been caused by the test material (see Fig. 1).

1. Aspirate the media after the incubation period is over.
2. Prepare the MTT solution by reconstituting the MTT powder in 1× sterile PBS to a final concentration of 5 mg/mL and

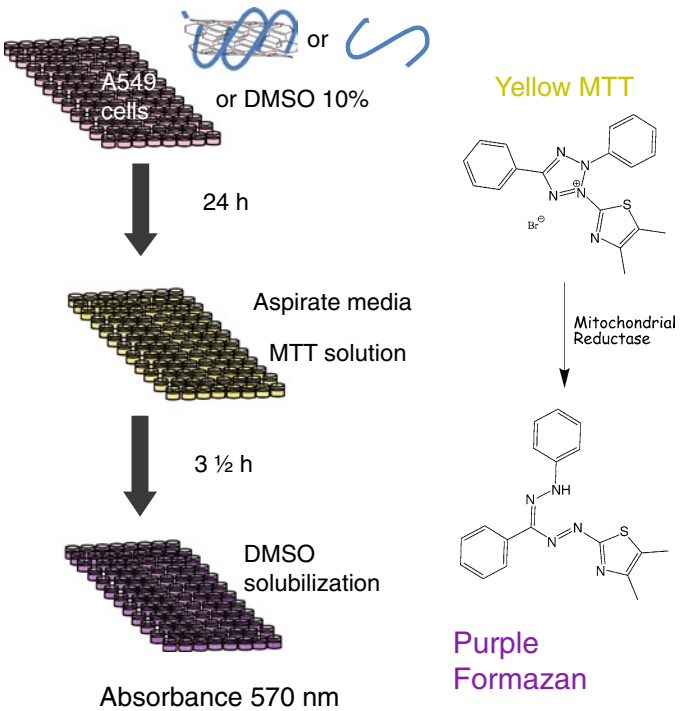

Fig. 1. Schematic of the MTT assay.

subsequently filter and sterilize this solution using a 0.22-μm sterile filter. Store in 2 mL aliquots at −20°C protected from light until use (stable for at least 6 months after reconstitution).

3. Dilute the MTT solution with complete media containing FBS (at a ratio of 1:6) and add 120 μL of this solution to each well.
4. Incubate the cells for 3.5 h at 37°C in a humidified atmosphere (5% CO_2) incubator.
5. Remove the MTT solution by gently inverting the 96-well plate into a paper tissue.
6. Add 150 μL DMSO (100%) to solubilize the formazan crystals and incubate the plate for 15 min at 37°C to remove air bubbles.
7. Read the absorbance at 570 nm in a plate reader and express the results as the percentage cell viability ($n = 8 \pm$ S.D.) compared to untreated control cells (see Fig. 2 and Note 5). The percentage cell viability is calculated using this formula:

$$\%\ \text{Cell viability} = \frac{A_{570\,\text{nm}}\ \text{of treated cells}}{A_{570\,\text{nm}}\ \text{of untreated cells}} \times 100.$$

3.3.2. Original LDH Assay (See Note 6)

LDH is a stable cytosolic enzyme that is released from the cell upon cell lysis. The LDH assay is based on quantitatively measuring released LDH using a coupled enzymatic assay, in which LDH plays a role in the conversion of a tetrazolium salt (INT) into a red soluble formazan product which then can be measured colorimetrically. The amount of LDH released is proportional to the number of lysed cells (15) (see Figs. 3 and 4).

1. Transfer 50 μL media containing released LDH from all wells into a fresh 96-well plate.
2. For maximum LDH release: Add 10 μL lysis solution (10×) for every 100 μL of fresh media. Keep the cells after treatment at 37°C for 45–60 min.
3. Centrifuge the plate at 240 × *g* for 4 min and supernatant into the fresh 96-well plate.
4. Thaw the assay buffer and warm to room temperature, while keeping it protected from light.
5. Transfer 12 mL assay buffer into one vial of substrate mix. Gently mix to dissolve the substrate mix, while keeping it protected from light. Both the assay buffer and non-used reconstituted substrate mix can be stored again at −20°C (see Note 7).
6. Add 50 μL reconstituted substrate mix to each well containing the transferred aliquots. Cover the plate with foil and incubate for 30 min at room temperature.

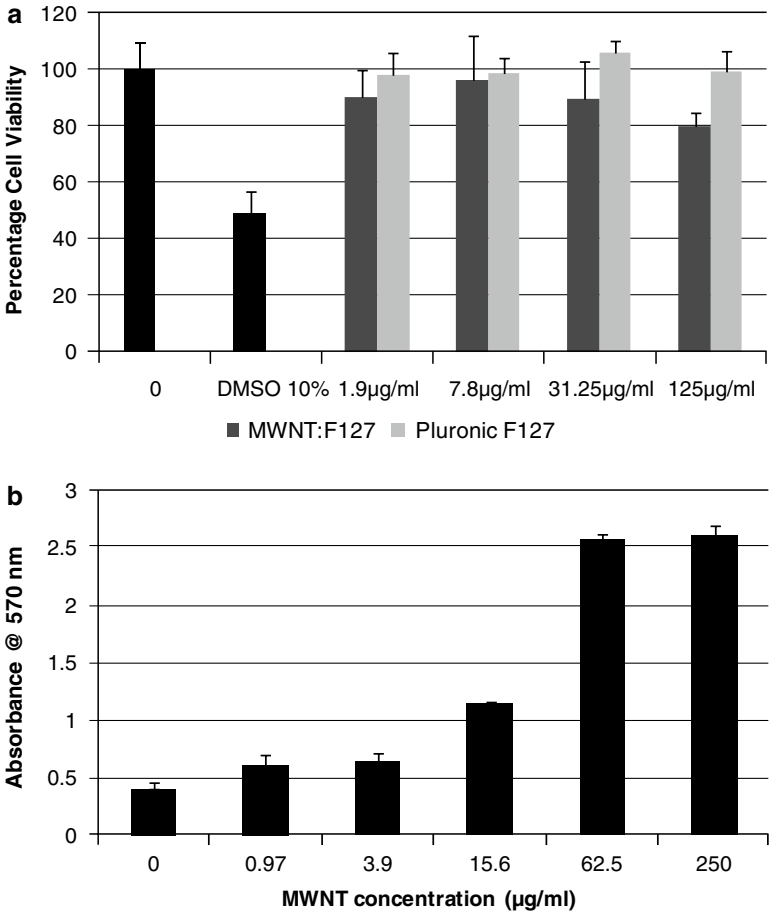

Fig. 2. (**a**) Percentage cell viability of A549 assessed by the MTT assay for varying MWNT and Pluronic F127 concentrations. DMSO is used as a positive control; untreated cells are the negative control (0). The MWNT:F127 dispersions show concentration-dependent toxicity after 24 h exposure. The values listed are the concentrations of MWNT in solution, the corresponding F127 control for each concentration is at a concentration ten times higher (e.g., 1.9 µg/mL MWNT, 19 µg/mL F127) (see Note 4). (**b**) Absorbance (at 570 nm) of insoluble formazan mixed with MWNT:F127 dispersions. The spiking experiment shows that the intrinsic absorbance of MWNT can interfere with the results of the MTT assay.

7. Add 50 µL stop solution to each well and pop any large bubbles using a syringe needle.
8. Read the absorbance of the solutions at 490 nm in a plate reader and express the results as the percentage LDH released ($n = 4 \pm$ S.D.) compared to maximum LDH released from the untreated control cells (see Fig. 5). The percentage LDH released (% cytotoxicity) is calculated using this formula:

$$\%\ \text{LDH released} = \frac{\begin{array}{c}A_{490\,\text{nm}}\ \text{of treated and untreated}\\ \text{cells} - A_{490\,\text{nm}}\ \text{of media alone}\end{array}}{\begin{array}{c}A_{490\,\text{nm}}\ \text{of maximum of untreated}\\ \text{cells} - A_{490\,\text{nm}}\ \text{of media alone}\end{array}} \times 100.$$

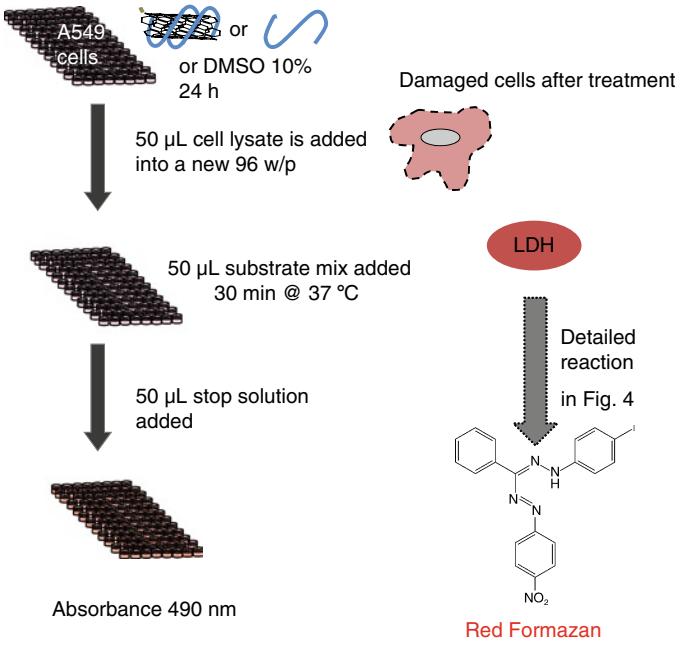

Fig. 3. Schematic of the original LDH assay.

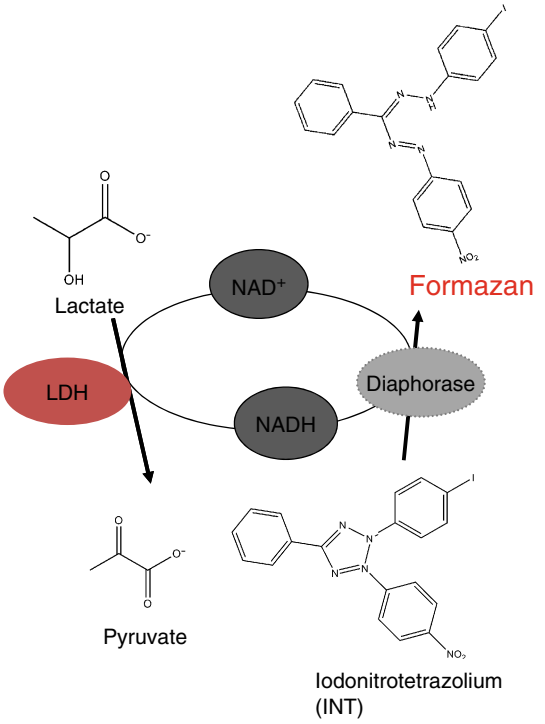

Fig. 4. LDH-mediated conversion of the INT salt into formazan.

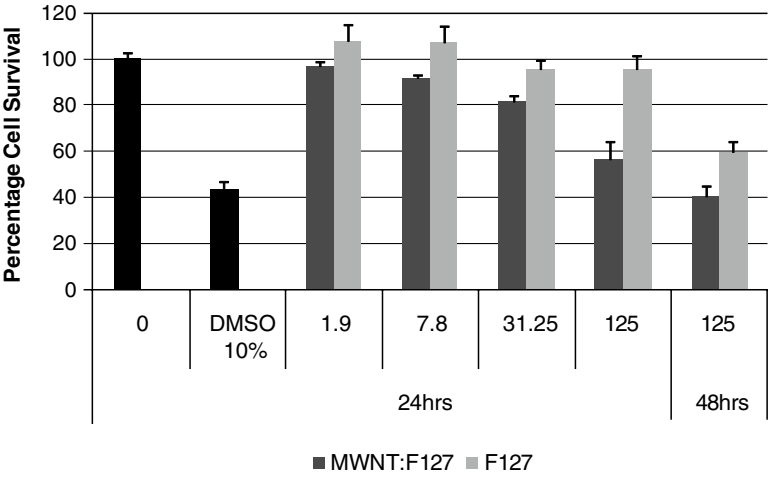

Fig. 5. Percentage cell survival with varying concentrations of MWNT:F127 and Pluronic F127. The values listed are the concentrations of MWNT in solution (in μg/mL), the corresponding F127 control for each concentration is at a concentration ten times higher (e.g., 1.9 μg/mL MWNT, 19 μg/mL F127) (see Note 4). DMSO was used as a positive control; untreated cells (0) were the negative control. MWNT:F127 showed a clear dose-dependent toxicity after 24 h of exposure, which was potentiated after 48 h due to Pluronic F127 toxicity. The Pluronic F127 did not, however, cause any cytotoxicity after 24 h incubation at the concentrations used.

The absorbance data at 490 nm also can be shown without converting it into percentage LDH release to highlight the interference of CNT with the results of the assay (see Fig. 6 and Note 8).

3.3.3. The "Modified LDH" Assay

The original colorimetric LDH assay was modified to avoid interference of the components used in the assay with the CNT. The survived cells after treatment are artificially lysed with Triton X-100, and the cell lysate is centrifuged in order to precipitate the CNT. The released LDH is therefore an indication of the number of viable cells that survived treatment with CNT (see Figs. 4 and 7).

1. Replace the media with 100 μL per well phenol and serum-free media (RPMI, phenol-free media) (see Note 6).
2. Add 10 μL 9% v/v Triton X-100 per 100 μL added phenol and serum-free media.
3. Incubate the plate at 37°C for 45–60 min (see Note 9).
4. Transfer the cell lysate into tubes and centrifuge at 16,000 × *g* for 5 min to pellet the uptaken CNT (see Note 10).
5. Transfer 50 μL cell lysate, avoiding the CNT pellet, into a fresh 96-well plate.
6. Add 50 μL reconstituted substrate mix to each well containing the transferred and centrifuged cell lysate. Cover the plate

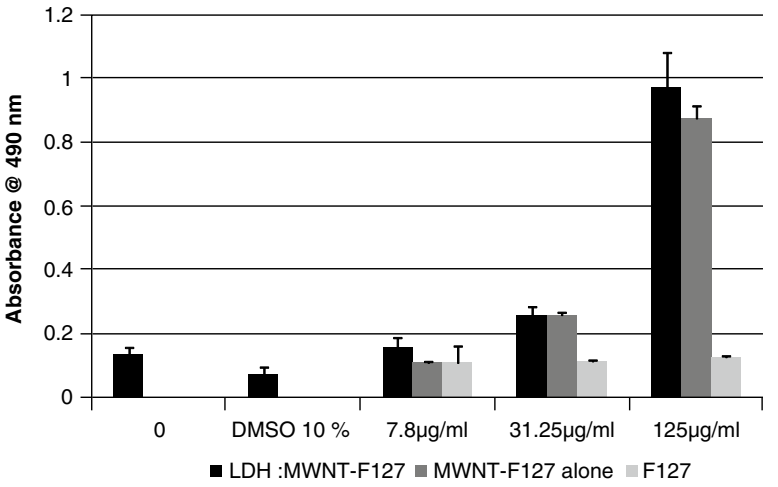

Fig. 6. Percentage LDH release (absorbance at 490 nm) after treatment of cells with different concentrations of MWNT:F127 and Pluronic F127. The values listed are the concentrations of MWNT in solution (in µg/mL), the corresponding F127 control for each concentration is at a concentration ten times higher (e.g., 7.8 µg/mL MWNT, 78 µg/mL F127) (see Note 4). MWNT:F127 dispersions (no assay) were used as controls (no assay). The absorbance of the released LDH in the MWNT-treated wells (LDH:MWNT:F127) is identical to the intrinsic absorbance of the MWNT:F127 which indicates that the observed LDH readings are attributed in part to the intrinsic absorbance of CNT. The LDH enzyme might also be inhibited by the presence of the positive control (DMSO 10%) as it shows low absorbance compared to the untreated control.

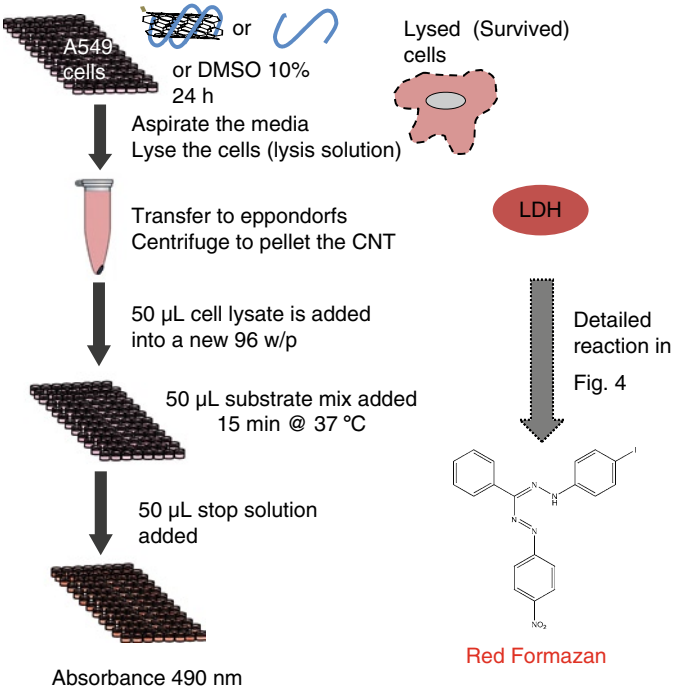

Fig. 7. Schematic of the modified LDH assay.

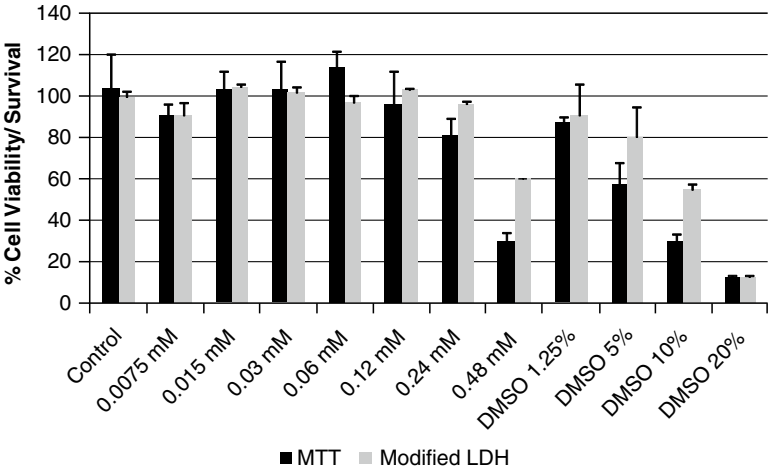

Fig. 8. Different concentrations of cationic liposomes (0.0075–0.48 mM) and DMSO (1.25–20%) were used to assess the reliability of the modified LDH version compared to the MTT assay. Very similar toxicity patterns were observed with both assays, emphasizing the reliability of using the modified LDH assay for the in vitro cytotoxicity assessment of CNT.

with foil and incubate for 15 min at room temperature. (Follow steps 4 and 5 from Subheading 3.3.2 for the preparation of the reconstituted substrate mix).

7. Add 50 µL stop solution to each well and pop any large bubbles using a syringe needle.
8. Read the absorbance at 490 nm in a plate reader and express the results as the percentage cell survival ($n = 4 \pm$ S.D.) compared to untreated control cells (see Fig. 8 and Note 11). The percentage cell survival is calculated using this formula:

$$\%\ \text{Cell survival} = \frac{A_{490\,\text{nm}}\ \text{of treated cells}}{A_{490\,\text{nm}}\ \text{of untreated cells}} \times 100.$$

4. Notes

1. Noncovalently functionalized CNT are used as an example. If desired, different dispersing agents or CNT that are modified with different surface molecules also can be used. If covalently functionalized CNT are used, disperse them in 5% dextrose by bath sonication for 30–45 min and store at 4°C until further use.
2. 9% v/v Triton X-100 in deionized water can also be used as a lysis solution.
3. A well-dispersed sample of MWNT should contain no precipitates or aggregates. If desired, SWNT can be used, however,

they are not as easily dispersed in F127 as MWNT; longer sonication times may be required. Concentrations higher than 1 mg/mL MWNT are not easily dispersed.

4. We used MWNT:F127 dispersions with MWNT concentrations ranging from 0 to 125 μg/mL. As you dilute the MWNT:F127 stock in cell culture media, the concentration of the Pluronic F127 is also diluted. So, in these samples, the F127 concentration varies as the CNT concentration is varied. As a result, the corresponding F127 controls should have concentrations in the range between 0 and 1,250 μg/mL.
5. The concentration-dependent decrease in cell viability (see Fig. 2a) should be viewed with apprehension. According to published data, the MWNT might adsorb to the formazan crystals (through a strong, π–π stacking interaction) and not allow them to dissolve when the solubilizing agent (DMSO) is added. This would cause a falsely low reading in the absorbance at 570 nm and falsely low percentage cell viability. Further, we believe that the intrinsic absorbance of CNT can alter the results of the assay when high cellular uptake and internalization of CNT occurs. We spiked the insoluble formazan with different concentrations of the MWNT:F127 dispersion and read the absorbance at 570 nm (see Fig. 2b). An increase in absorbance was observed as the concentration of MWNT increased indicating that the intrinsic absorbance of the MWNT is also contributing significantly to the absorbance at 570 nm. This effect results in falsely high cell viability, explaining why during many assays cell viability over 100% is obtained. The systematic effects of these two different types of interference can have the opposite effect on the results of the assay causing unreliable and irreproducible results. In conclusion, the use of the MTT assay for the assessment of CNT cytotoxicity should be avoided due to this unpredictable balance between false-positive and -negative readings.
6. Media containing phenol and serum (FBS) can contribute to background absorbance. This background should be subtracted from all results before calculating the percentage LDH released. In order to reduce this background without affecting cell viability, use phenol-free media with a reduced serum concentration (5%). If a positive control is desired for this LDH assay, gently vortex the LDH positive control and mix 2 μL into 10 mL PBS + 1% bovine serum albumin (BSA) (1:5,000 dilution). This stock should be prepared fresh before each use and triplicate or quadruplicate wells are recommended. This control should have an absorbance of 1.39 ± 25% at 490 nm.
7. Reconstituted substrate mix can be stored for 6–8 weeks at – 20°C without loss of activity. Upon storage, a precipitate

may occur in the assay buffer which can be removed by centrifugation at 300 × *g* for 5 min and does not affect the assay performance (15).

8. The original LDH assay, similar to the MTT assay, is a colorimetry-based method therefore significant CNT interference is possible. As can be seen from Fig. 6, the media containing the released LDH showed exactly the same absorbance at 490 nm as MWNT:F127 dispersions diluted in media. Therefore, it is not possible to attribute the trend seen in Fig. 6 (as the concentration of the MWNT was increased, there was an increase in the LDH released) to actual cytotoxicity. Note that the positive control (DMSO 10%) showed low LDH release (low absorbance) compared to the other controls. This could be due to the inhibition of LDH enzyme by the DMSO in the media.
9. This step can be replaced by a freeze–thawing cycle. Incubate the plate at –70°C for approximately 30 min followed by thawing at 37°C for 15 min and then proceed to step 4.
10. It can be difficult to precipitate the CNT from the media due to their high dispersability. If necessary, the centrifugation time can be increased depending on the amount of CNT uptaken by the cells. A discernible pellet should be observed at the end of the centrifugation step. The centrifugation speed does not seem to affect the release of LDH over a range of speed from 300 to 16,000 × *g*. Centrifugation at 4°C is preferable since the LDH enzyme is stable at 4°C.
11. The toxicity of cationic liposomes, which do not interact with the chemicals used in such assays, was analyzed using this modified protocol and the MTT assay. Results of both assays showed the same trends, proving that the modified version of the LDH assay is in fact promising and reliable (see Fig. 8).

Acknowledgments

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