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In vitro methods for Nanotoxicity Assessment: Advantages and Applications

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ABSTRACT

Nanotechnology is the production of materials at atomic and molecular level and is expected to open some new avenues to fight and prevent diseases. It leads to improvement in biology, biotechnology, medicine and healthcare by uncovering the structure and function of biosystems at the nanoscale. The size of nanomaterials is similar to that of the most biological molecules and structures; therefore, nanomaterials can be useful for both in vivo and in vitro biomedical research and applications. Due to the expected growth in this field and new materials being employed, there is a call for safety and exposure risks. Hence, for improved characterization and reliable toxicity assessments, toxicological studies of nanosystems are growing at exponential rates annually. For these reasons, screening assays are needed to assess the chemical and physical properties of nanomaterials. Lacking the proper interactions of nanostructures with the biological systems, it is unclear whether the exposure could produce harmful biological responses. Deploying these materials in vivo has even more challenges. So, in vitro methods are commonly used for toxicity assessment of nanoparticles. Nanoparticle risk assessment can be done with existing cytotoxicity methods, or with the development of new test systems with new standards for a general in vitro toxicity testing of nanoparticles. An altogether different approach is required for nanoparticle characterization and for bioassays, in order to validate their properties in physiology. The present review focuses on the various in vitro methods of nanotoxicity assessment and the advantages offered by them. The article also sheds some light on the applications of these methods.

Keywords: Nanotechnology, Nanotoxicity, Nanomaterials, *In vitro* methods.

INTRODUCTION

Nanotechnology is the technique through which structures with size ranging between 1 and 100 nm are developed, which imparts them unique properties [1].Owing to their unique properties at this size level, there is a rapid expansion of nanotechnology in scientific, technical and commercial field. The new and unique applications offered by nanotechnology in diverse areas have made it so popular, that it is being applied today in almost all aspects of

daily life. A number of products having nanosize elements are available in the market with still new more to come [2]. As a result, there is an increasing demand for raw nanomaterials, which can range from nanosized metals and metal oxides to carbon nanotubes for fulfilling the growing needs of the market. [3,4]. In view of an increase in manufacturing and consumer utilization of nanoparticles, there is a release of these materials into the environment, eco-system, water [5] and food supplies, and the other routes of non-voluntary entry into the human body [6]. According to conservative estimates [7], more than 800 consumer products containing nanoparticles or nanofibers are already in the market, and a number of others are still to come. According to "The Nanotechnology Consumer Products Inventory" [8], the most common material mentioned in the product descriptions was carbon (29 products), which included fullerenes and nanotubes. Silver was the second most referenced (25 products), followed by silica [14], titanium dioxide [7], zinc oxide [7], and cerium oxide [1].

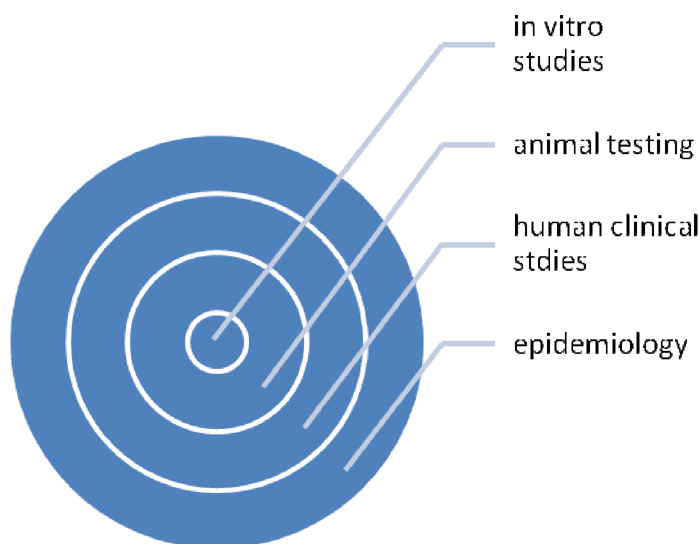
With the growth of nanomaterials in scientific field as well as in technical field, there is an increasing exposure of nanomaterials to humans, together with the distinct properties like complex interactions, possible bioaccumulation, unique chemistry and physical parameters. All of these properties mandate development and validation of accurate nanodevice and materials characterization protocols, which are capable of predicting toxic as well as hazardous reactions. These methods must reliably predict and assess the possible outcomes of effects, from benefits to possible risks, and health hazards associated with exposure to nanomaterials, as they become more widespread in manufacturing and medicine. The inter-agency National Toxicology Program classifies the new entity with its data along with their possible risks associated with the entity. After that the entity is interrogated by a set of tests which are basically designed to characterize a given risk, and also to characterize the mechanisms for related outcomes [9].With the ongoing commercialization of nanotechnology products, human exposure to nanoparticles will dramatically increase, and an evaluation of their potential toxicity is essential. A number of manufactured nanoparticles have recently been shown to cause adverse effects *in vitro* and *in vivo* [10–12]. The nanomaterials have some unusual physiochemical properties due to their small size, chemical composition, surface structure, solubility, shape, and aggregation [13] .Owing to the lack of understanding of the size, shape, composition and aggregation-dependent interactions of nanostructures with biological systems [14], it is not confirmed whether the exposure of humans, animals, insects and plants to engineered nanostructures could produce harmful biological responses [15, 16]. Hence, a new sub-discipline of nanotechnology called nanotoxicology has emerged.

Nanomaterials characterization is important since nanoparticles might interact with assay components or interfere with detection systems, resulting in unreliable data [17]. There are a number of different approaches that can be taken to assess the toxic effects of inhaled complex mixtures, including air pollution particles. These include epidemiology studies, human clinical studies, animal studies, and *in vitro* studies. Each of these approaches has its own strengths and advantages. Various studies suggest that *in vitro* nanotoxicity data can reduce the testing of animals by identifying an appropriate starting dose for *in vivo* studies, and a limited amount of toxic waste is produced [18]. .*In vitro* methods can be used to estimate toxicokinetic parameters and target organ toxicity, thereby, increasing the predictions of toxicity, and reducing animal use for some tests under controlled testing conditions [19]. However, many of the necessary *in vitro* methods for this program have not yet been developed. Other methods have not been evaluated for reliability and relevance, and their usefulness and limitations for generating information to meet regulatory requirements

for acute toxicity testing have not been assessed. Risk assessment of complex mixtures is the most accurate and defensible, when as many of these approaches as possible, can be used in an integrated manner to address a specific question [20].

This review, briefly reflects on the utility and advantages of various *in vitro* assays in nanotoxicology, provides an overview of currently used *in vitro* cytotoxicity methods, and furthermore, it discusses general applications of *in vitro* methods that may provide new approaches to nanoparticle risk assessment. These methods are specifically discriminatory to nanoscale properties, sizes or physical states, and many do not report sensitive information about the nanomaterial behaviours in biological systems. These assays are important in characterizing nanomaterial applications in biotechnology, ecosystems, agri- and aqua-culture, biomedical applications and toxicity screening.

Figure: 1 Role of *in vitro* studies in pharmacology and toxicology studies



Merits of *in vitro* systems:

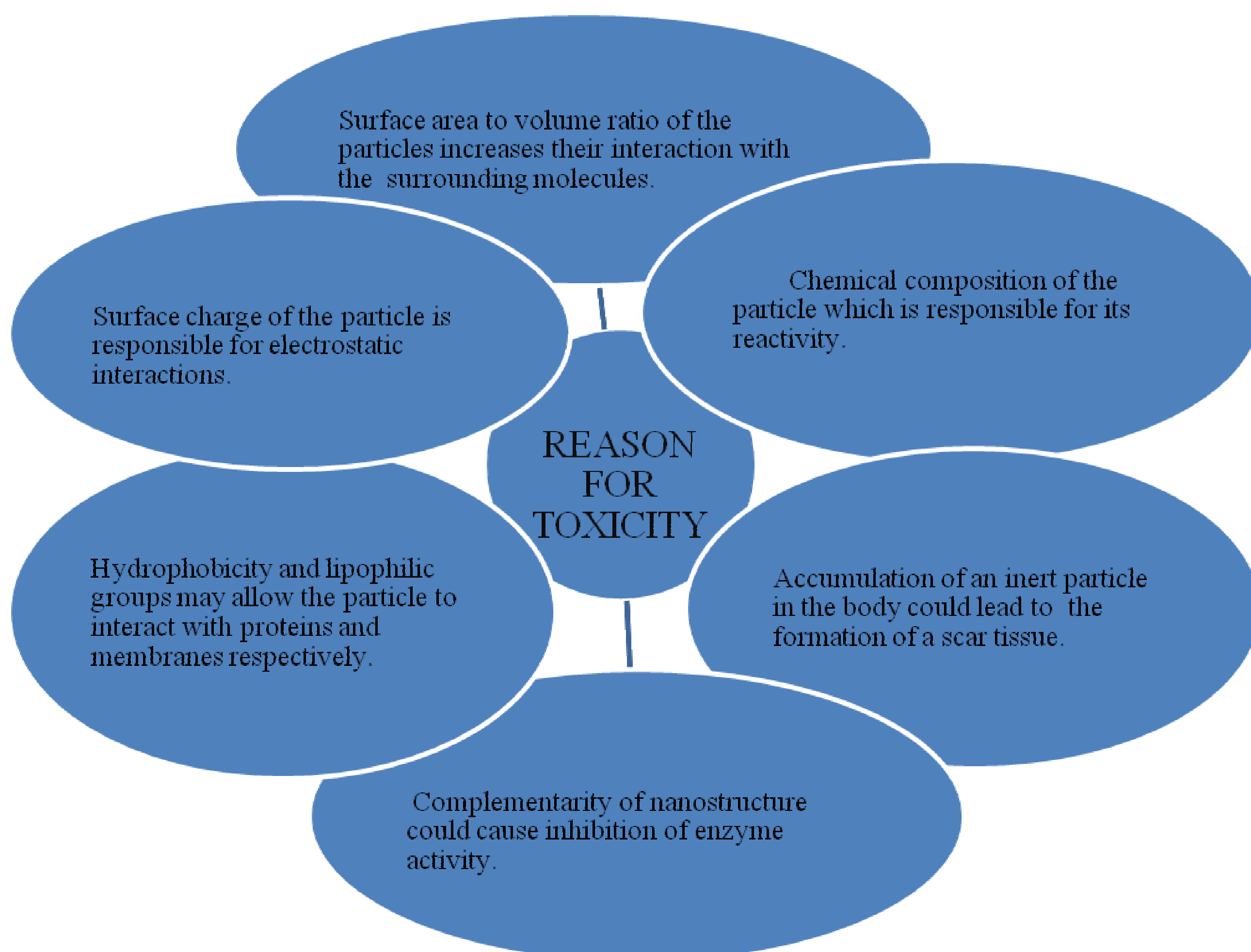
In vitro toxicological assessment is an important tool for nanotoxicology. The various merits of these systems are as follows:-

- These systems are performed under controlled testing conditions in a particular environment.
- There is reduction in systemic effects by using these systems.
- Reduction of variability between experiments.
- The same dose range can be tested in a variety of test systems (cells and tissues).
- Time-dependent studies can be performed and samples taken.
- Testing methods are fast and cheap.
- Very small amount of test material is required.
- Limited amount of toxic waste is produced.
- *In vitro* methods can be performed using human cells and tissues.
- Transgenic cells carrying human genes can be used.
- Reduction of testing in animals [21].

Need for acute toxicity testing
Internationally, the most common use of acute systemic toxicity data is to provide a basis for hazard classification and the labelling of chemicals for their manufacture, transport, and use (**Organisation for Economic Cooperation and Development, 1999a**). The OECD guidelines set out how governments expect companies to behave. They offer a basic outline for corporate codes of conduct on how to deal with socially relevant issues. Other potential uses for acute toxicity testing data include:

- Establish dosing levels for repeated-dose toxicity studies;
- Generate information on the specific organs affected;
- Provide information related to the mode of toxic action;
- Aid in the diagnosis and treatment of toxic reactions;
- Provide information for comparison of toxicity and dose response among substances in a specific chemical or product class;
- Aid in the standardization of biological products;
- Aid in judging the consequences of exposures in the workplace, home, or from accidental release, and serve as a standard for evaluating alternatives to animal testing.

Figure: 2 Factors responsible for toxicity due to nanoparticles



General *in vitro* methods for nanotoxicity assessment

1) Cell viability assay:

A) Proliferative assay:- These are the mainly metabolic assays which include:-

Tetrazolium salts assay, which measures the viability of a cell population relative to control, untreated cells [22]. Cells are treated with particulates for various times before addition of soluble yellow tetrazolium salts such as MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3 carboxymethoxyphenyl)-2- (4-sulfophenyl)-2H-tetrazolium, inner salt) or MTT (3-(4, 5-dimethylthiazol-2- yl)-2, 5-diphenyltetrazolium bromide) for 2-4 hr at 37°C. During this process, viable cells with active respiratory mitochondrial activity bioreduce MTS or MTT into an insoluble purple formazan product, via mitochondrial succinic dehydrogenases, which is subsequently solubilized by dimethyl sulfoxide (DMSO) or detergent, and quantitated on a visible light spectrophotometer[23,24]. Data are represented as optical density (OD)/control group. This technique has many advantages when compared to other toxicity assays because it requires minimal physical manipulation of the model cells and yields quick, reproducible results, requiring simple optical density acquisition [25]. However, this assay has a number of drawbacks such as, certain human cell lines are inefficient at processing the tetrazolium salt reagents, and the requirement of DMSO to solubilize the formazan product generated by reduction of the tetrazolium salts is problematic. In addition, it exposes the laboratory personnel to potentially hazardous amounts of solvent [26]. As a result, a number of modifications have been established, including the use of the tetrazolium derivative XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide), which is metabolized to a water soluble formazan product, thereby, eliminating the solubilization step required with MTS or MTT [26-28].

Alamar Blue has been relatively recently applied to nanotoxicological studies by assaying cellular redox potential. AlamarBlue is a proven cell viability indicator that uses the natural reducing power of living cells to convert resazurin to the fluorescent molecule, resorufin. The active ingredient of alamar blue (resazurin) is a nontoxic, cell permeable compound that is blue in color and virtually non-fluorescent. Upon entering the cells, resazurin is reduced to resorufin, which produces very bright red fluorescence. Viable cells continuously convert resazurin to resorufin, thereby, generating a quantitative measure of viability—and cytotoxicity [29]. The redox indicator is non-toxic to cells, users and the environment. It also produces a clear, stable and distinct change, which is easy to interpret.

Incorporation of [3H] thymidine into the DNA (deoxy ribonucleic acid) is a sensitive measurement of cell proliferation. The use of [3H] thymidine is complicated due to *in vitro* toxicity and expensive radioactive material, and requires special training and facilities. Moreover, this technique often requires a lengthy incubation period (24-48 hr) with [3H] thymidine [30]. This method has been used to demonstrate the ability of nitric oxide-releasing nanofiber gels to inhibit vascular smooth muscle cell proliferation *in vitro* [31]**.**

Cologenic assays: Interactions between nanomaterials and probe molecules can be avoided altogether through the use of cologenic assays [32, 33] .The cologenic assay allow studying the effectiveness of specific agents on the survival and proliferation of cells. After plating at a very low density, cells are allowed to grow until colonies are observed, and then, cells can either be pre-treated with particulates of interest, or treated following plating. It is assumed that each colony originates from a single plated cell which can be stained with crystal violet

or nuclear stains, where colonies of highly proliferating cells are counted by visual inspection.

B) Apoptosis assay: - Apoptosis, a form of programmed cell death have been used extensively during nanotoxicological research, and include inspection of morphological changes, comprising various assays which are as follows:-

DNA laddering, the oldest DNA damage assay technique, characterizes this fragmentation by isolating and fluorescently labeling DNA from cells exposed to a potential toxicant in culture. DNA damage is then detected by gel electrophoresis.

Caspase assays are based on the measurement of zymogen processing to an active enzyme and proteolytic activity [34]. As soon as Caspase-3 is activated, cell death is inevitable. Activated Caspase-3 can be detected by measuring the cleavage of a Caspase-3 substrate linked to a chromophore or fluorophore that absorbs or emits light when separated from the substrate [35].

The Comet Assay, also called single cell gel electrophoresis is a sensitive and rapid technique for quantifying and analyzing DNA damage in individual cells. Individual cells are embedded in a thin agarose gel on a microscope slide. All cellular proteins are then removed from the cells by lysing. The DNA is allowed to unwind under alkaline/neutral conditions and then DNA undergoes electrophoresis, allowing the broken DNA fragments or damaged DNA to migrate away from the nucleus. After staining with a DNA-specific fluorescent dye such as ethidium bromide or propidium iodide, the gel is read for amount of fluorescence in head and tail, and the length of tail. The extent of DNA liberated from the head of the comet is directly proportional to the amount of DNA damage [36].

TUNEL assay, which derives its name Terminal deoxynucleotidyl transferase dUTP(deoxy uridine triphoshate)nick end labeling relies on double-strand breakage, like the damage necessary for DNA fragmentation during apoptosis. TUNELassay is based on incorporation of biotinylated nucleotides conjugated to bromodeoxyuridine (BrdU) at the 3' OH ends of the DNA fragments that form during apoptosis. This detection system utilizes a biotin conjugated anti-BrdU antibody and streptavidin-horseradish peroxidase [37].

Annexin V which is regularly used to detect apoptotic cells [38] binds strongly to phosphatidylserine in a calcium-dependent manner [39]. Phosphatidylserine is normally excluded from the extracellular side of the plasma membrane [40], but flips between the inner and the outer side upon the onset of apoptosis [41]. Fluorescently labelled Annexin V can, therefore, be used to detect apoptotic cells.

C) Necrosis assays:-This includes following assays:-

The Neutral red uptake cytotoxicity assay procedure is a cell viability assay based on the ability of viable cells to incorporate and bind neutral red, a weak cationic supravital dye that readily penetrates cell membranes by non-ionic diffusion, and predominately accumulates intracellularly in lysosomes, with lysosomal fragility and other changes that gradually become irreversible [42]. Cytotoxicity is expressed as a concentration dependent reduction of the uptake of neutral red after chemical exposure, thus, providing a sensitive, integrated signal of both cell integrity and growth inhibition.

In trypan blue assay cells are treated with agents, trypsinized, and subsequently stained with trypan blue, a diazo dye, which is taken up by dead cells, but excluded by viable cells. Unstained cells reflect the total number of viable cells recovered from a given dish. This method is advantageous because it conveys the actual number of viable cells, and increases or decreases in comparison to control, untreated cells.

LDH is a soluble cytosolic enzyme which serves as an indicator of lytic cell death. The colorimetric lactate dehydrogenase (LDH) assay which is based on the oxidation of the yellow tetrazolium salt, INT, to a red formazan, has a long tradition in the clinic to evaluate tissue or cell damage [43]. As significant amounts of LDH are released from the cytosol upon cellular necrosis, LDH activity is measured in the cell culture supernatant.

2) Oxidative Stress Assay:-

Oxidative stress is defined as excess formation and/or insufficient removal of highly reactive molecules, due to the disturbance in the oxidative balance by engineered nanoparticle,s such as reactive oxygen species (ROS), and reactive nitrogen species (RNS). ROS include free radicals such as superoxide (•O_2-), hydroxyl (•OH), peroxyl (•RO_2), hydroxyperoxyl (•HRO2-), as well as, non-radical species such as hydrogen peroxide (H_2O_2) and hydrochlorous acid (HOCl). RNS include free radicals like nitric oxide (•NO) and nitrogen dioxide (•NO2), as well as, non-radicals such as peroxynitrite (ONOO-), nitrousoxide (HNO_2) and alkyl peroxynitrates (RONOO). The generation of abnormally large concentrations of ROS and RNS can have many toxicological implications, by reaction with proteins, lipids or nucleic acids, leading to abnormal cellular function [44].

In **2, 7-dichlorofluorescin (DCFH) assay,** the dye is obtained as a diacetate precursor, which is cleaved by high pH to make the non-fluorescent product DCFH [45]. The presence of ROS converts DCFH to a fluorescent product, 2, 7-dichlorofluorescein, which can be measured by fluorimetry.

Electroparamagnetic resonance (EPR) is also a technique that has been widely used to assess nanoparticles and particle- induced ROS generation. The use of specific spin traps or probes in combination with specific reagents can allow for the quantification, as well as, specific identification of the free radical species generated. For EPR detection of radicals, an adduct-forming, spin-trapping agent (5,5-dimethyl-lpyrroline N-oxide, DMPO) for hydroxide (OH^-) or superoxide (O^{2-}) radicals or a radical-consuming spin probe (4-hydroxy-2,2,6,6-tetramethylpiperidine- 1-oxyl) are introduced into the culture or nanoparticle solution, for a set amount of time, after which the entire supernatant is collected, vortexed, and analyzed on an EPR spectrometer[46,47].

Lipid peroxidation is the oxidative degradation of cell membranes initiated by the presence of ROS, and is most commonly measured by assaying the presence of malondialdehyde or other thiobarbituric acid reactive substances [48-50]. This assay has been used extensively to demonstrate the ability of a variety of nanomaterials to elicit lipid peroxidation in multiple cell types, such as: fullerenes in human dermal fibroblasts (HDF) and human liver carcinoma (HepG2) cells [49].

The plasmid assay has been used to assess ROS production [51]. In this assay, unwinding and linearization of a coiled bacterial DNA plasmid is used to estimate free radical and/or

ROS exposure. This technique is not particularly sensitive, and may be subject to DNA binding to the nanoparticle surface.

Oxidative stress acts by alterations in superoxide dismutase or glutathione production. Increases or decreases in these responses can be interpreted as an evidence for oxidative stress, as the cell either compensates for increased stress by upregulating the production of antioxidants, or the exhaustion of cellular stores of superoxide disumutase (SOD), or glutathione (GSH) by oxidation from RNS or ROS. GSH is an essential antioxidant that is oxidized during oxidative stress to form a GSH-GSH disulfide between two GSH molecules yielding GSSG. The most quantitative assessment monitors the ratio of GSH and its disulfide oxidative product GSSG using HPLC [50], but chromatographic separation steps are time-consuming and allow for auto-oxidation, leading to over-estimation in the amount of GSSG. For this reason, combined GSH and GSSG have been assayed instead, during the nanotoxicology studies to date, using 5, 50-dithio-bis(2-nitrobenzoic acid) (DTNB)[52].The total GSH concentration is determined by the colorimetric detection of 5-thio-2-nitrobenzoic acid after reaction of DTNB with GSH. SOD activity is determined indirectly via the inhibition of superoxide oxidation of a colored substrate, nitro blue tetrazolium, where superoxide is generated via exogenous xanthine-xanthine oxidase[53].

3) Inflammatory Assay:-

Enzyme-linked immunosorbent assay (ELISA), is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample. In ELISA, an unknown amount of antigen is affixed to a surface, and then a specific antibody is applied over the surface so that it can bind to the antigen. This antibody is linked to an enzyme, and in the final step a substance is added, that the enzyme can convert to some detectable signal, most commonly a colour change in a chemical substrate. The most commonly tested human and murine inflammatory markers are the chemokine Interleukin-8 (IL-8), followed by TNF-α and IL-6[54].

Current *in vitro* methods used in nanotoxicity assessment and their advantages:

As with any other man-made materials, both *in vitro* and *in vivo* studies on biological effects of nanoparticles should be performed. Presently, in absence of any clear guideline(s) by the regulatory agencies on the testing/evaluation of nanoparticulate materials, *in vitro* studies (using established cell lines and primary cells derived from target tissues) become extremely relevant and important. These *in vitro* model systems could provide a rapid and effective means to access nanoparticles for a number of toxicological endpoints, allow development of mechanism-driven evaluations, and provide refined information on how nanoparticles interact with human cells in many ways. In fact, elaborate *in vivo* studies on experimental laboratory animals are mandatory before any clinical trials especially involving human subjects. Nevertheless, *in vitro* methods with their advantages are preferred and conducted prior to animal experimentation and clinical trials. Assessment of defined toxicity endpoints by *in vitro* methods is more rapid and economical, as compared to, animal models. Complexity of selection of appropriate animal models or the human body is not a problem with *in vitro* test system, and the metabolic activity of standardized cell lines has often not been comprehensively characterized.

Table 1: *In vitro* methods and their advantages

Assay	Detection Principle	Purpose	Advantages	Example of assay effect	Used for nanoparticles	Refernce
Tetrazolium salts (MTT, MTS, XTT, WST)	mitochondrial activity is determined colorimetricaly and by visible light spectrometer	Cell viability/cell growth (Cell metabolic activity)	1)Real time assay results using low cell numbers 2)Provides simple method for estimation of live cell number in order to assess rate of cell proliferation and to screen cytotoxic agents [55] . 3)Non radioactive 4)Inexpensive	1)Increased cytotoxicity of thiolated gelatine nanoparticles designed to release their contents in a reducing environment[49] 2) Long circulating monensin nanoparticles (LMNP) were shown to potentiate the *in vitro* cytotoxic effects of anti-My9, a ricin-based immunotoxin, in HL-60 sensitive (500x potentiation) and resistant (5x potentiation) human tumour cell lines [56].	Silver nanoparticles	[57] [58]
					carbon nanoparticles	[59][60] [27]
					Fullerenes	[61][26]
Neutral red assay	Colorimetric detection of intact lysosomes and detected via fluorescence or absorption measurement.	Cell viability (Lysosomal activity)	1) Quantitative estimation of the number of viable cells in a culture. 2) One of the most used cytotoxicity tests with many biomedical and environmental applications [62].	The neutral red uptake (NRU) in NIH3T3 mouse fibroblasts is the only validated *in vitro* method for toxicity testing [15] and has been incorporated into the REACH (Registration, Evaluation, Authorisation and Restriction of Chemical substances)for the *in vitro* toxicity assessment of chemicals[63].	Carbon nanotubes,	[28] [64]
					Silver, molybdenum, aluminum, iron oxide and titanium dioxide nanoparticles	[57]
Lactate dehydrogenase (LDH)	Detection of LDH release colorimetrically	Cell viability	Reliability, speed and simple evaluation	1) Nanoparticles containing different metal/metal oxide groups have recently been analyzed by the LDH assay for their toxic effects on rat liver BRL3A cells [65]. 2) LDH release studies were conducted on human lung epithelial (16HBE14o) cells	Carbon nanoparticles	[26]
					ZnO (zinc oxide) nanoparticles	[66]
					Fullerenes	[67]
					Iron Oxide nanoparticles	[65]

				treated with nanoparticles consisting of porcine gelatin and human serum albumin		
Trypan blue	Detected either colorimetrically or fluorescently	Cell viability/cell growth	1)It conveys the actual number of viable cells and increases (cell proliferation) or decreases (cytotoxicity) in comparison to control, untreated cells	1) Cytotoxicity of crocidolite asbestos as well as other minerals including talc and glass beads on a TERT-1 immortalized, contact-inhibited human mesothelial cell line, LP9/ TERT-1[68]. 2) Poly (lactic) acid nanoparticles (PLA) for gene delivery in human and bovine retinal pigment epithelial cells, do not reduce cell viability at concentrations up to 4 mg/ml PLA [69].	Gold nanoparticles	[70]
					SWCNT (single-walled carbon nanotubes)	[71]
Colony formation Assay	Detected microscopically or by scanner	Proliferative capacity	1)Reliable determination of the number of cells required to distinguish between a cluster of cells and a colony 2) It enables rapid and accurate enumeration of colony number and is more suitable for higher throughput compound assessment than current microscope based methods. 3) This approach determines colony number through the application of a volume algorithm and permits the differentiation of cytostatic effects	1)Cytotoxicity in A549 cells exposed to medium depleted by two types of SWCNT in order to determine if these carbonaceous nanoparticles are capable of reducing the availability of medium components[72]	Carbon based nanomaterials	[73]
Caspase-3 activity	Fluorimetric detection of Caspase-3 activity	Apoptosis	1)Easy, fast and more convenient 2)Potent, cell permeable and	Nanoscale HAP(hydroxy, when administered to human gastric cancer	Silver nanoparticles	[57][58] [77]

			non-toxic fluorochrome inhibitor 3)A direct measure of apoptosis expressed as the number of active caspase enzymes present in the cell 4)No need for cell lysis no membrane permeabilization	cells (SGC-7901) at 100 µg/ml for 12-48 hr, caused release of cytochrome c and activation of caspases-3 and -9 [74]. Finally, it has been demonstrated that both CeO_2 (5-40 µg/ml) and TiO_2 (5-40 µg/ml) nanoparticles trigger the activation of caspase-3 in Beas-2B cells following 24 hr of exposure [75, 76].		

Applications

1) Novel application of an *in vitro* technique to the detection and quantification of botulinum neurotoxin antibodies e.g detection of Clostridium botulinum [BoNT] neutralising antibodies is currently achieved using the mouse lethality assay [MLA] [96].
2) *In vitro* techniques are used for the assessment of neurotoxicity [97].
3) Attempts are being made to use this technique to establish new varieties from chimeric tissues e.g rooted cuttings of *Chrysanthemum morifolium* cv. Maghi, a small flowered, late blooming cultivar, were treated with different doses of gamma rays. Somatic mutations in flower colour (light mauve, white, light yellow and dark yellow) and chlorophyll variegation in leaves were detected as chimeras in treated populations [98].
4) *In vitro* methods are used to select highly susceptible individuals among common squirrel monkeys (Saimiri sciureus) to bacterial lipopolysaccharides by using peripheral whole blood [99].
5) *In vitro* techniques are used to forage germplasm [100].
6) Applications of *in vitro* methods to Eucalyptus germplasm conservation [101].
7) A potential diagnostic application of magnetization transfer contrast: an *in vitro* NMR study of excised human thyroid tissues [102].
8) Application of *in vitro* methods for selection of Lactobacillus casei strains as potential probiotics[103].
9) *In vitro* models are also used for Antioxidant Activity Evaluation [104].
10) *In vitro* methods are also used to determine dermal corrosivity of chemicals [105].
12) *In vitro* methods can also be applied for detecting cell-mediated immunity in man [106].
13*) In vitro* methods used to assess the nutritive value of leaf protein concentrate [107].

CONCLUSION

Nanotechnology is the manipulation of structures at molecular level. Owing to its vast growth in every field, be it biotechnology, agriculture or commercial field, it is necessary to study its chemical and physical properties, and characterize these nanomaterials according to them. Due to diverse nature of nanotechnology, there are significant challenges in the interpretation, validation and correlation of cell and tissue toxicity data collected for nanomaterials. Advances in nanotoxicology will come from developing a valid set of reliable toxicity tests and nanomaterial characterization protocols for application to variety of nanomaterials that have been produced, and the even greater variety that is yet to come. The unique challenges in nanotoxicity assessments lie in addressing the current lack of

appropriate tools to directly observe and interrogate nanomaterials in complex biological systems. Specifically, materials aggregation, physical, and chemical reactivity are nearly impossible to understand currently. Significantly, pharmacological dose–response relationships are complicated by time- and condition-dependent nanomaterial chemical and physical states. Acute versus chronic nanomaterial exposure effects and hazards are, therefore, difficult to monitor. Hence, multiple different measurement techniques must be adapted, carefully assessed for validity, and applied to complex nanomaterial systems. Nanomaterial toxicities in biological systems present unique and complex problems. Hence, *in vitro* methods are commonly used to determine nanotoxicity. These methods are advantageous as they minimize the need for animal testing and can be performed under controlled testing conditions.

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