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Important issues in the cytotoxicity screening of nano-sized materials

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ABSTRACT

Due to their extraordinary properties nano-sized materials (NMs) are increasingly used in industrial, pharmaceutical and medical applications. An even broader use is currently limited by concern about their potential adverse effect on health. Screening for toxic effects of all engineered NMs therefore, is needed to demonstrate biocompatibility. The identification of adverse cellular effects is one of the first steps in the toxicological assessment of drug compounds before they get to the market. A panel of cytotoxicity screening assays is available and can be used also for the assessment of NMs. The use of these established and validated assays for the testing of NMs, however, is complicated by the fact that NMs may interfere by color, chemical reactivity and light scattering leading to false positive or false negative results. The paper illustrates the principles of conventional cytotoxicity screening assays and discusses their suitability for the assessment of NMs. Adequate controls to identify interference and alternatives, if interference with the used assay is seen, are suggested.

INTRODUCTION

Nano-sized materials (NMs) have revolutionized the industrial, chemical, pharmaceutical and medical market due to their surprising properties. The use of NMs improved the chemical and physical characteristics, efficiency and durability of many products. On the other hand, it was also realized that NMs differ from the bulk materials they were made from regarding toxicity. The higher penetration through organ barriers and the great surface area of the small particles resulting in a high reactivity and the generation of reactive oxygen species appears to be one main reason for this effect ([1]). This finding forced the researcher to screen and characterize the biological actions of their engineered NMs. For the assessment of toxic effects on cells a panel of cytotoxicity screening assays (CSAs) is available, which was originally developed for the pre-clinical testing of drug compounds intended for medical use. As no specific guidelines are available for the testing of NMs, these CSAs are generally used for the assessment of nanotoxicity. This article will mention specific problems related to cytotoxicity testing of NMs. It aims to help the researcher in the interpretation of data, in the identification of interference and to suggest alternative testing methods.

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CSAs are able to identify any influence on cell metabolism and viability by the assessment of cell functions common to all cell types like proliferation, organelle function and cell death. They are termed basic cytotoxicity assays and differ from cell-specific cytotoxicity assays, which assess specific cellular functions. Basal CSAs are performed on cell lines, whereas for specific CSAs generally primary cells are better suited because most cell lines are either derived from cancer cells, where specific cell functions are partly lost or their ability to produce cell-specific products is lost during the immortalization process to generate the cell line. Cell lines from various cell types can be obtained commercially and are well characterized. They show higher reproducibility of growth and response to stimuli than primary cells. Primary cells show variation in quality due to isolation and difference in the donor and they can not maintained in culture for prolonged time. They retain, however, more characteristics of this cell-type and are used when cell-specific functions are assessed.

Table I: Principles of detection of the most commonly used cytotoxicity screening assays

Determination	Example	Mode of read-out
Cell number	Coulter counter, CASY Technology xCELLigence®	Electrochemical sensing Impedance measurement
Protein content	Sulforhodamine B staining, Leucine incorporation	Colorimetric Radiometric
DNA content	PicoGreen*	Fluorometric
Metabolism	ATP-content	Luminescent
Enzyme activity	Dehydrogenase activity (formazan bioreduction, Resazurin), Esterase activity	Colorimetric, fluorometric Fluorometric
Organelle function	Neutral Red uptake	Colorimetric

* there are many other nuclear dyes like for instance Hoechst, SYTO and TOTO dyes to stain nuclei but PicoGreen has the advantage of binding only to double strand DNA and not to single strand DNA and RNA.

Induction of apoptosis, disruption of membrane integrity, inhibition of proliferation and inhibition of specific cellular processes or organelle function change the signal in CSAs. Most CSAs are based on determination of cell number (cell counting), of cellular products and of organelle function (Table I), where vital and viable cells contribute to the signal. The method used should be suitable for the detection of vital cells independent of the cell type. Techniques like for instance impedance measurement can not be used for all adherent cells ([2]) and some techniques, like detection by ATP-content, may be more sensitive than others ([3]) but in general all CSAs are suited for the detection of cytotoxicity. Vitality indicates the metabolic status of the cell (see scheme Fig. 1a). Several dyes are available to identify enzyme activities; they turn fluorescent or colored upon metabolization (Fig. 2b). In the case of viability, which reflects plasma membrane integrity, dye uptake can only occur, if the plasma membrane is damaged. Viable cells are identified by absence of staining (see scheme Fig. 1b and example in Fig. 2b). As most CSAs do not differentiate between vital and viable cells, like for instance quantification of cellular protein (Fig. 2a), the more general term 'viability' is commonly used.

a

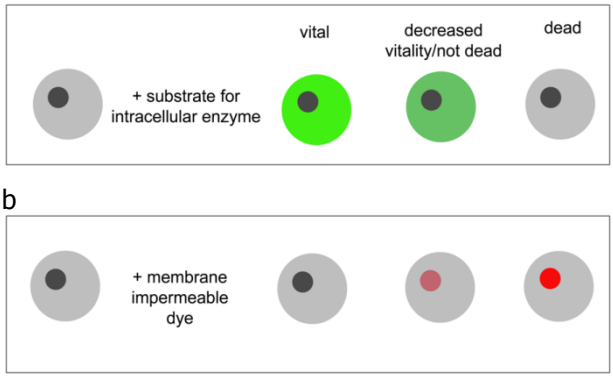
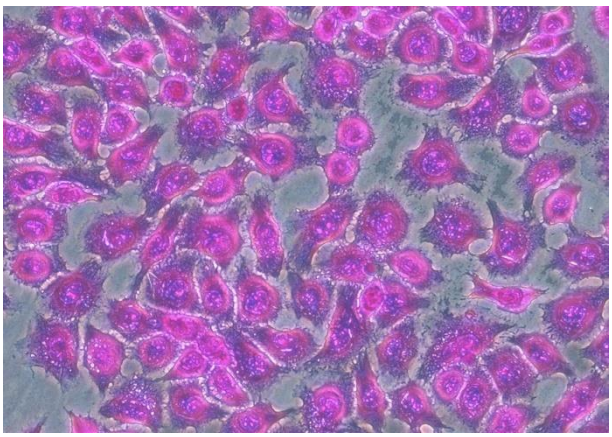


Fig. 1: Principle of CSAs. a: The addition of a membrane permeable substrates for intracellular enzymes, like for instance tetrazolium salts or calcein acetoxymethyl ester, identifies vital cells by strong fluorescence. b: Addition of a membrane-impermeable dye, which stains the nucleus by intercalation with the DNA like propidium iodide or ethidium homodimer 1, labels dead, non viable cells.

CSAs have been used for drug compound testing for many years producing reliable results with a high correlation of IC50 values (half-maximal inhibiting concentration) in cell lines to LD50 values (concentrations in blood killing half of the individuals, [4]).

A



B

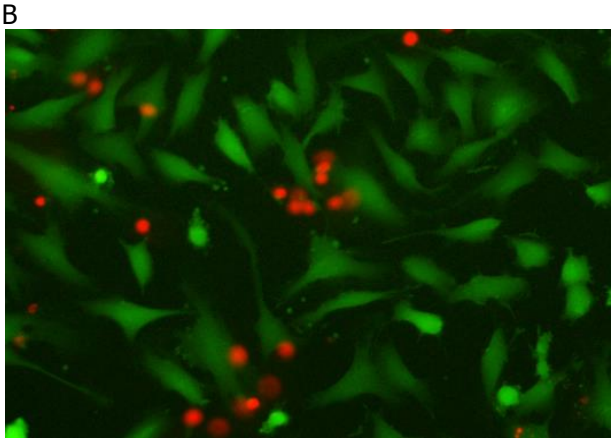


Fig. 2: Example for detection of protein by sulforhodamin B staining (pink, A). The intensity can be assessed by photometry. Example for the combined vitality/viability detection using metabolization of calcein acetoxymethyl ester (green, B) for vitality and ethidium homodimer 1 (red) uptake for loss of membrane integrity.

In contrast to conventional drug compounds NMs may cause problems in CSAs. Examples for interference caused by NMs comprise conversion of tetrazolium salts ([5-7]), adsorption of dyes ([8, 9]), interference by absorbance ([10, 11]), by fluorescence ([9, 12]), by binding of proteins ([13]), by dye degradation ([14]), by redox reactions ([15]) and by light scattering ([16, 17]).

Nanoparticle properties like high adsorption capacity, hydrophobicity, surface charge, optical and magnetic properties, or catalytic activity may interfere with assay components or detection systems, Carbon nanotubes, C_{60} fullerenes, carbon black, quantum dots, nanodiamonds, silver particles, iron oxide and mesoporous silica particles may interfere with CSAs ([8, 9, 18-22]).

The basic principles in the testing can be adopted from the CSA of conventional drug compounds. For the detection of cytotoxicity adherent cells like epithelial cells and fibroblasts are preferred to cells growing in suspension like blood cells. The first reason is that NPs most often are designed for treatment of adherent cells and regenerative medicine, where epithelial cells and fibroblasts, both adherent growing, are the most relevant cell types. Testing of compatibility in blood (hemocompatibility) is a later point in the toxicological assessment and includes in addition to cytotoxicity also function of red and white blood cells and effects on coagulation. For the testing adherent cells additionally have the advantage that adhering NMs, which could interfere with the read out, can be removed by washing after the exposure. Generally, a 96-well plate format is adequate for the testing. We usually use evaluation of 4h and 24h and use the template illustrated in Fig. 3. If NMs can not be dispersed directly in cell culture medium, but need a specific solvent, untreated cells are also cultured in medium + solvent. Evaporation takes place at peripherally located wells and induces slightly different growth of cells in the center and the periphery of the plate.

Table II: Testing in case of assay interference

Problem	Action	Example
SC ≠ untreated cells	Use other solvent, lower concentrations	EtOH -> DMSO
CC ≠ GC	Use an assay where cells are washed or where only supernatant is used	ATP-content -> sulforhodamine staining
IC ≠ B	Use another read-out, another reaction principle	Formazan bioreduction -> ATP-content
IC50 at 4h = IC50 at 24h	Use another read-out, another reaction principle	Formazan bioreduction -> ATP-content

SC: cells + medium with solvent in the concentration of the highest sample concentration; CC: cells + medium + NMs, without assay compounds; GC: cells + medium; IC: NM + medium + assay compounds, without cells, B: medium.

Wells at the periphery can be used for medium, blanc (cell culture medium without cells) and NM specific controls but are less suitable for the testing of samples. Controls for interference with assay compounds and with the readout (NMs with assay compounds but in the absence of cells) and for interference by adherence to the cells (cells and NMs in the absence of assay compounds) are

included. We use particles, non conventional drug compounds, as positive and negative controls.

Abnormal reaction in one of the controls, most often in IC and CC, and identical curves at different time points are indicative for interference. When interference of NMs with the assay is identified several actions can be taken to obtain a more meaningful result (Table II): a CSA with a different reaction principle (metabolism instead of enzyme activity), with a different read-out (fluorometric instead of colorimetric) and with different treatment of the cells (evaluation of a plate-adherent product instead of analysis of the supernatant) can be used. If possible cells should be checked for morphology to confirm the findings. Assays based on radiometry can also be biased by NMs and, therefore, are not the solution for all problems.

CSAs are often used in combination with assays for membrane integrity. Per definition, cytotoxicity is the result of cell damage; induction of apoptosis and inhibition of proliferation are not cytotoxic events because they also occur in specific physiologic situations. Assays investigating membrane integrity use either the detection of intracellular enzymes in the supernatant of cultured cells or the uptake of cell impermeable dyes into the cells. Common examples for the former principle are lactate dehydrogenase or adenylate kinase detection. For dye-uptake the fluorescent dyes propidium iodide (Fig. 2b), ethidium homodimer 1, Z-aminoactinomycin D, TO-PRO-3, TOTO-3, SYTOX dyes, which all bind to DNA, are used. Alternatively, detection by color with trypan blue and eosin can be performed. The combination of CSAs and membrane integrity assays can be used for the identification of interference because viable cells produce opposite signals in both assays. If for instance a given NM interacts by light absorption the signal would increase in both assays indicating normal vitality in the one assay and compromised membrane integrity in the other assay.

If interference is seen with all CSAs available to the researcher the comparison of two time points may help.

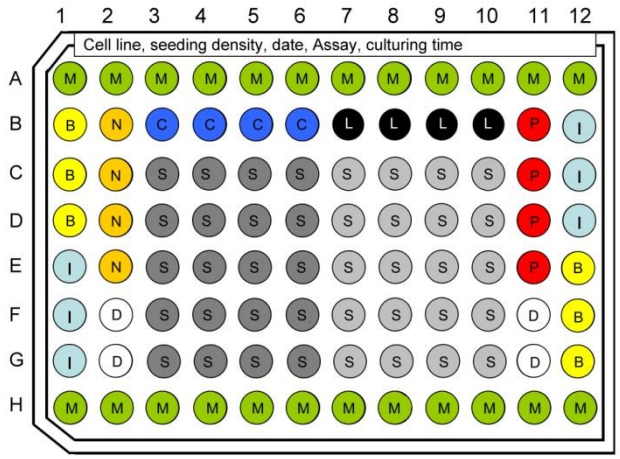


Fig. 3: Layout for CSA including medium to prevent evaporation (M), blank (B, medium), dispersant control (D, cells + medium + dispersant), interference control (I, medium + NM + assay compounds, without cells), positive control (P, cells + medium + particles with cytotoxic effect), negative control (N, cells + medium + particles with no cytotoxic effect), color control (C, cells + medium + NMs, without assay compounds), lysis (L, lysed cells only for membrane integrity assays) and two samples at different concentrations including untreated cells (S).

With longer incubation times an increase in cytotoxic action is expected. Under the assumption that interference with the NMs does not increase in the same degree but remains constant cell damage can be extrapolated by comparing the changes between the different time points.

CONCLUSION

Several CSAs with different detection principles, both in read-out and in reaction mechanism, should be used for the assessment of NMs. Although some NMs show interaction with many CSA most NMs do not pose major problems in CSA testing. When comparing toxicity data from different groups assessed by a given CSA it should not be neglected that composition of the culture medium, use of dispersant or other additives and the proliferation rate and the type of the cells used in the assay also have a profound influence on the result ([23]).

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