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Interference of engineered nanomaterials in flow cytometry: A case study

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ABSTRACT

Nanotechnology is regarded as the enabling technology of the 21st century. However, only a relatively small number of nano-enabled medical and healthcare products finally made their way to the market. There are several reasons why such innovative approaches fail in translation, with one key factor being the uncertainty surrounding their safety assessment. Although well described, interference reactions of engineered nanomaterials (ENM) with classical cytotoxicity assays remain a major source of uncertainty.

Flow cytometry is a powerful, widely used, *in vitro* technique. Its readout is based on the detection of refracted laser light and fluorescence signals. It is therefore susceptible to ENM interference. Here we investigated possible interferences of ENM in the Annexin V/propidium iodide (PI) assay, which quantifies apoptotic and necrotic cell populations by flow cytometry.

Two case studies were conducted using either silica or gold nanoparticles differing in size, specific surface area and surface chemistry. Both ENM types were found to cause distinct interference reactions at realistic concentrations. Silica particles induced false-positive signals; however only in the absence of a protein corona and in conjunction with a particular fluorophore combination (FITC/PI). In contrast, gold particles led to complex quenching effects which were only marginally influenced by the presence of proteins and occurred for both fluorophore combinations analyzed. We present a versatile spike-in approach which is applicable to all ENM and cell types. It further allows for the identification of a broad range of different interference phenomena, thereby increasing the reliability and quality of flow cytometry and ENM hazard assessment.

1. Introduction

Engineered nanomaterials (ENM) have a huge potential in industrial and healthcare applications. Even though the number of publications in this area has been exponentially increasing over the last years, only a few products have found their way onto the healthcare market. One reason is the lack of reliable testing strategies that are acceptable to regulatory bodies for proper risk assessment. Although there have been efforts to address this in recent years at the European level like ITS-Nano and NanoREG, the issue of a standardized hazard characterization for ENM is still not solved.

Ideally, a limited number of simple tests, preferentially *in vitro* or even better *in silico,* would reliably classify any possible adverse effect of a given (nano)material towards human beings and the environment. Even though it is not that easy and straight forward, evaluating whether or not a given ENM poses a risk for human health under defined circumstances is indeed the overall goal for a safe and sustainable use of ENM. Of course, no single approach or assay will be able to answer such a complex question. Therefore, a multi-parameter testing strategy, as depicted in Fig. 1, is needed. Important pillars of such a testing strategy are (i) the physico-chemical characterization (PCC) of the ENM of interest as well as its behavior in specific (ii) *in vitro* but also (iii) *in vivo* settings. Compiling the results from all pillars provides the "big picture" of ENM effects according to a given objective, with respect for example to a particular material and its possible exposure route(s). Each pillar comprises several parameters such as the determination of ENM size or surface charge, which, among others, are examples for PCC. Within the *in vitro* assessment pillar, acute cytotoxic events can be addressed according to the ROS paradigm [1–7]. Therefore relevant endpoints could be: (i) ROS overproduction and cellular oxidative stress reactions, (ii) acute cytotoxicity, (iii) inflammatory reactions as well as (iv) genotoxicity. Each of these endpoints is ideally assessed by two perpendicular assays of different natures. Accordingly, systematic or assay intrinsic errors e.g. due to ENM interferences (see below) can be

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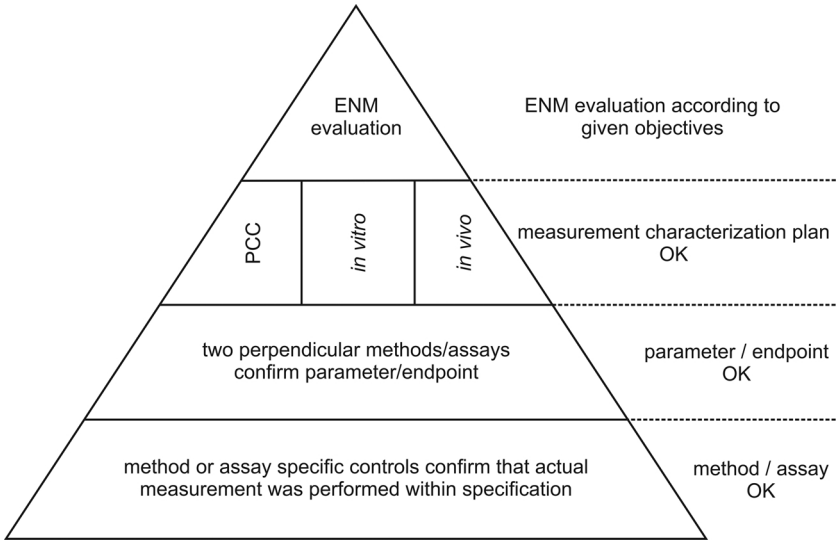

Fig. 1. Strategy for ENM evaluation. PCC = physico-chemical-characterization.

recognized, and in the best case even avoided. Finally for each and every assay, it is of great importance to ensure that the results obtained are valid and reliable. One prerequisite to achieve that is the implementation of assay specific positive and negative controls confirming that the actual measurement was performed within specification [8]. Furthermore, interference controls are needed to identify false positive and/or false negative results due to interactions of the ENM with assay components. The pyramid shown in Fig. 1 illustrates that only if each and every assay delivers valid and reliable results, is the entire ENM hazard assessment built on solid grounds.

One important aspect of reliability is to minimize sources of variability in the respective assay. Sources of variability can be manifold even for simple *in vitro* cytotoxicity assays as elaborated in detail by Roesslein and co-workers [9]. These authors made use of cause-and-effect analysis as a powerful tool to summarize and visualize the potential sources of variability in the MTS assay. They even went two steps further in that they deduced relevant controls and implemented the respective experiments into a 96-well plate layout. In an inter-laboratory comparison study, the same group of scientists was able to confirm the applicability of the approach and its suitability for daily use [10].

The sources of variability detailed by Roesslein et al. [9] included general aspects of good cell culture practice, and are thus of importance for any cell-based assay. Besides that, assay specific parameters have to be taken into consideration. Finally, the impact of ENM on measurement results has proven to be an important aspect of reliable assay performance [8,9]. Convinced by this approach, we considered the published general aspects for this study, and adapted the assay specific points focusing particularly on ENM interference phenomena. This was done for another common cytotoxicity assay: the Annexin V/PI assay that is used to quantify apoptotic and necrotic cells by flow cytometry. Flow cytometry is a powerful method, widely used in cell biology, which allows for the fast screening of diverse cell populations. Its readout is based on the scattering of light and/or the detection of specific fluorophores, such as for example Annexin V-FITC and PI. It has been shown that depending on their physico-chemical properties, ENM can interfere with fluorescence dyes as well as in absorbance measurements commonly used in cell-based cytotoxicity assays. Even though such interference phenomena have been described [11–18] no general approach is available that provides a rapid, cheap and easy way to exclude ENM interferences in flow cytometry. Although it might not be possible to eliminate or circumvent interference by all types of ENM, it is nevertheless, of great importance to identify false-positive/negative results due to interference.

To systematically address ENM interference in the flow cytometric Annexin V/PI assay, we chose two model ENM known for their broad applicability in medical as well as consumer products, namely silica (SiO_2-NP) and gold (Au-NP). The selection of SiO_2-NP differed in size and specific surface area whereas the set of Au-NP differed in surface functionalization, which is of importance in the nanomedical field. Here, we introduce a versatile strategy to detect the occurrence of ENM interference. It further allows determining the maximum concentration of ENM up to which reliability of cellular measurements is ensured. Beyond that we elaborate on potential modes of action and ways to circumvent certain interference reactions. This general approach is applicable to any cell type and any ENM.

2. Materials and methods

2.1. ENM

Synthesis and characterization of all ENM used is described in the Supplementary Information (SI). A summary of particle nomenclature and relevant properties is shown in Table 1.

2.2. Cell culture

Cell lines were obtained from ATCC (Manassas, VA, USA) and maintained at 37 °C and 5% CO_2 in humidified atmosphere and routinely sub-cultured twice a week at 70–80% confluency by treatment with 0.5% trypsin-EDTA (Sigma-Aldrich or Welgene for A549 or HepG2, respectively).

A549 cells (ATCC® CCL-185™) were cultivated in Roswell Park Memorial Institute (RPMI) 1640 Medium (Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS, Sigma-Aldrich), 1% L-glutamine (Sigma-Aldrich) and 1% penicillin-streptomycin-neomycin (PSN, Sigma-Aldrich). HepG2 cells (ATCC® HB-8065™) were cultivated in Dulbecco's Modified Eagle's Medium (DMEM, Welgene) supplemented with 10% FCS (Welgene) and 1% penicillin-streptomycin (PS, Welgene).

2.3. Flow cytometry and Annexin V/PI assay

For detection of apoptotic and necrotic cells the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences) and the PE Annexin V Apoptosis Detection Kit I (BD Biosciences) was used. Cells were seeded