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Navigating towards improved cytotoxicity assessment in nanomedicine development: Shifting from colorimetric to fluorescence-based assays

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It is well known that the characterization of nanomedicines can pursue different levels of complexity, both in the development stage and in the quality control process [1]. In line with physicochemical aspects, even more obstacles are encountered in biological safety assessment, while anticipation of their immunogenic potential represents an additional challenge. Moreover, interactions between the test reagents and the nanomaterial have been identified as one of the most important issues in toxicity testing that influence market authorization of nanomedicines, which ought to be resolved [1]. The European Nanomedicine Characterization Laboratory – the reference laboratory for nanomedicines, provides protocols for 2 colorimetric cytotoxicity assays employing LLC-PK1 (porcine kidney epithelial cells) and Hep-G2 (human hepatocarcinoma cells) cell lines. However, the latest recommendations in the field underline the demand for enhancing the testing procedures, while proposing incorporation of immune cells as target cell lines for toxicity evaluation, aiming to provide more reliable conclusions on nanomedicine safety in preclinical level.

In this study 2 inherently different types of pharmaceutical nanosystemes were selected: nanoemulsion (NE) and solid lipid nanoparticles (LNP) and subjected to a set of orthogonal toxicity evaluation assays. Adjusted WST-1 (assessing mitochondrial activity as an indicator of cellular well-being) and LDH (lactate dehydrogenase release evaluation as an indicator of cell membrane damage) assays have been performed as the colorimetric tests, while propidium-iodide (PI)-based assay was developed as a fluorescent counterpart (able to directly distinguish live from dead cells), using RAW 246.7 cell line (murine macrophages – immune system cell line). Starting concentration of the tested nanoformulations was 50 % v/v, and they were subsequently diluted with the factor of 2, to create a total of 8 concentrations. Incubation time was 4 h.

Presented assays rely on completely different biological bases. Therefore, their careful combination can address some shortcomings in the *in vitro* evaluations established so far. Although similar toxicity trends were observed regardless the assay used, it was evident that the LDH assay required specific consideration. Since the supernatant is the subject of the analysis (not the cells directly), containing not only the enzyme of interest, but also the nanoformulations, in the wells corresponding to the 3 highest concentration of the NE/LNP pronounced scattering effects were observed. Such an event could be easily overlooked, potentially affecting the conclusions. However, it was overcome by careful design of control and blank wells (each test concentration was coupled with its own blank well containing no cells, but the same concentration of the NE/LNP in the culture medium). In contrast, absorbance measurements in WST-1 assay were performed in the absence of the NE/LNP, avoiding any interactions or scattering effects. Finally, developed PI-based assay proved to be the most relevant method. Based on the penetration of PI into the dead cell only, attaching to their DNA, the concentration of the dead vs. live cells could be directly estimated. What is more, after the incubation time, the measurements can be performed in the nanoformulation-free environment, surpassing the potential interactions. Notably, cell viability obtained in the PI-based assay followed the same trend as in the WST-1 assay but with significant difference in the obtained values for the first 3 concentrations (Figure 1).

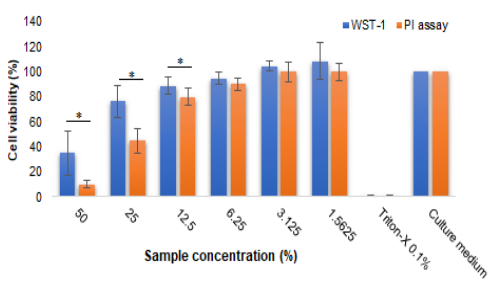

Figure 1. Cytotoxicity assay results for the NE: WST-1 and PI-based assay (*p<0.05)

[1] Simon, *2023. J Control Rel*, 354, 120-127.
[2] Bremer et al., 2016. Publications Office of the European Union.

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