

# Cytotoxic potential of nanoliposomes on hCMEC/D3 and SH-SY5Y cell lines

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## Introduction

It is well known that nanoliposomes (NLs) are considered as pharmacologically inactive vesicles, exhibiting minimal toxicity. Despite their biocompatibility and cell membrane structure similarity, it is important to highlight that their potential cytotoxicity is tightly related to the cell culture model, exposure time and dose as well as NLs physicochemical and surface properties including lipid composition, size, surface charge, PEGylation and others (Inglut et al., 2020; Syama et al., 2022).

Therefore, the aim of this study was to investigate the cytotoxic potential of different formulations of NLs on two different cell culture lines

## Materials and methods

### Materials

Immortalized human cerebral microvascular endothelial (hCMEC/D3) cell line and Human neuroblastoma cell line (SH-SY5Y) were purchased from CELLutions, Biosystems/Cedarlane®, Canada and LCG Standards, Germany, respectively. CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (MTS) and CytoTox-ONETM Homogeneous Membrane Integrity Assay (CytoTox) were obtained by Promega, USA.

Both cell culture lines were seeded and cultivated according to the Manufacturers' guidelines.

### Preparation of NLs

NLs samples (SL:CH:PEG = 8.71:1:0; 8.71:1:1.67 and 8.71:1:0.67 molar ratios for NLb0, NLb1 and NLb2,

respectively) were prepared by modified lipid film hydration technique in details elaborated in Shalabalija et al. (2021).

### Cell viability assay (MTS assay)

*In vitro* cell viability studies were performed on hCMRC/D3 and SH-SY5Y cell lines after treatment with NLb0, NLb1 and NLb2. Cells at a density of  $1 \times 10^4$  cells/well were seeded in 96-well plates, (200  $\mu$ /well) (Cellstar® 96 well Cell Culture Microplate, Greiner Bio-One GmbH, Germany) (200). Cells were then incubated for 24 h (achieving confluence of around 80%), medium was then removed and cells treated with different NLs formulations to reach final concentrations of 5, 10  $\mu$ g/mL to 100  $\mu$ g/mL. After 24 hours of incubation with NLs, the medium was removed and further processed for LDH assay. Cells in each of the wells were incubated with MTS solution (20  $\mu$ L, 4 hours, 37 °C and 5% CO<sub>2</sub>). Absorbance was measured at 490 nm on a microplate reader (FLUOstar, BMG LabTechnologies, Germany). Cell viability was calculated as % of cell alive in respect to the positive control (cells not treated with NLs).

### Cell cytotoxicity assay (Lactate dehydrogenase assay - LDH assay)

After 24-hour incubation of the NLs with the respective cell lines in the previously mentioned range of concentrations, the medium was removed. A volume of 25  $\mu$ L was transferred to a white 96-well plate (Corning® 96 Well Solid Polystyrene Microplate). and 25  $\mu$ L of substrate (CytoTox-ONETM) was added. After the incubation step

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(20 min, 37 °C and 5% CO<sub>2</sub>), 13 µL of stop solution was added to each well. The amount of LDH released in the supernatant, as a result of the cell membrane damaged, was detected by fluorescence measurement using a microplate reader at 560 nm excitation and 590 nm emission (FLUOstar, BMG LabTechnologies, Germany). Cytotoxicity was calculated as a percentage of the amount of LDH released from the cells in the presence of Triton X-100 (positive control).

## Results and discussion

### *Cell viability assay (MTS assay)*

Obtained results showed that the cell viability of hCMEC/D3 cell culture line over a period of 24 hours was 90.92±6.43% (NLb0), 85.89±4.80% (NLb1) and 99.54±7.44 to 107.41±7.12% (NLb2) in the tested concentration range 5-100 µg/mL. When referring to the neuronal cell culture line, the metabolic activity of the cells was ~90% for NLb0 and NLb1, while cell viability >95% was characteristic for the wells treated with all concentrations of NLb2. Therefore, obtained data suggest all three formulations in the three tested concentrations showed decrease in the cell viability for <15%, indicating on the slight effect of the NLs on the cell metabolic activity on the both cell culture lines.

### *Cell cytotoxicity assay (LDH assay)*

With the aim to gain more detailed information regarding the potential toxicity of the NLs and also to ensure whether the exposure time and dose are safe for conducting cell uptake experiments, the next step of this research was the evaluation of the activity of the released cellular LDH in response to cellular damage. The performed experiments provided information that when the both cell lines were exposed at concentrations of 5 and 10 µg/mL of all three formulations, the levels of released LDH (5.654±1.25 - 23.54±3.44 and 4.52±1.13 - 13.33±32% for hCMEC/D3 and SH-SY5Y, respectively) was comparable low as the non-treated control group of cells (8.64±1.37 for hCMEC/D3 and 7.26±3.38% for SH-SY5Y), implying that the fusion and the uptake of the NLs did not affect the maintenance of the cell membrane integrity (Mihailova et al., 2021). On the other hand, it can be observed that there was a concentration-dependent increment in the percentage of released LDH for both cell lines. This increase is especially notable after treating the cells with the highest concentrations (100 µg/mL) of the NLs. In this sense, hCMEC/D3 showed 28.36±4.15, 8.56±2.85 and 26.04±4.34%, while SH-SY5Y was characterized by 23.55±3.55, 9.56±1.95 and 19.26±4.56% of cytotoxicity after 24-hour exposure at 100 µg/mL NLb0, NLb1 and NLb2, respectively. Additionally, it can be observed that

on both cell lines, the nano-carriers (NLb1) with the highest amount of PEG (50 mg) onto their surface exhibit with lowest cytotoxic potential, followed by NLb2 (5 mg PEG) and the non-PEGylated formulation (NLb0). This is in accordance with the literature data which suggests that PEG 2000 at certain concentrations is capable for fusion of single cells *in vitro* and participates in the processes of joining the membranes of adjacent neurons and axons. Additionally, PEG participates in sealing damaged neuronal membranes and enhancing their functional recovery through improving their capacitance, permeability and stability. The molecular mechanisms of these actions are still unclear, but it is believed that PEG chains tend to dehydrate the membranes, thus enabling their structure components to resolve and rearrange in a lamellar configuration followed by rehydration (Shi et al., 2013).

## Conclusion

Based on the obtained results, the three formulations did not show a decrease in the cell viability of both cell culture lines, nor a disruption of the integrity of the cell membrane after a 24-hour time period, which is pivotal for further determination of the therapeutic dose of the active substances encapsulated in these nano-carriers.

## References

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