Liposome Loaded Doxorubicin Delivery to Cells via Sonoporation

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Introduction. Sonoporation is a promising method designed to deliver biomolecules (drugs, DNA, RNA and proteins) to cells and tissues. The biophysical mechanism of molecular sonotransfer into cells is associated to microbubble (MB) stable and/or inertial cavitation. Oscillating and/or collapsing MBs evoke microstreams and/or microjets, which mechanically alter cell membrane permeability, thus, facilitating biocompound intracellular delivery.

It has been shown that sonoporation can be successfully exploited as anticancer treatment modality. Various cytotoxic drugs (cisplatin, bleomycin, mitoxantrone and doxorubicin) have shown promising prospects when used for both cancer cell viability decrease *in vitro* and tumour volume reduction *in vivo*. However, if these drugs are directly injected into the bloodstream they induce significant side-effects with cardiotoxicity and nephrotoxicity being of the primary importance [1]. In order to resolve this issue, the methods for side-effect diminishment are under extensive elaboration. One of the most promising methods is to use liposomes (LPs) (fluid filled lipid bilayer vesicles), loaded with anticancer drugs and directly attached to MBs [2].

Since we have already shown sonoporation to be an effective tool for the efficient delivery of anticancer drug doxorubicin (DOX) to cells [3], in this study we aimed to develop DOX loaded LPs and test, whether sonoporation can facilitate LPs loaded DOX delivery into cells.

Materials and methods. Chinese hamster ovary (CHO) cells were cultivated under the standard conditions [4]. For LPs preparation, 10 mM concentration dipalmitoilglicerofosfocholin (Avanti Polar Lipids, USA) and disteroilglicerofosfoetanolamin (Avanti Polar Lipids, USA) lipid solutions in chloroform (Sigma Aldrich, USA) were mixed to the ratio 3:1. The chloroform was removed by evaporation with nitrogen gas. The obtained lipid film was sonicated in amonium phosphate buffer (250 mmol/l; pH=7.4) at room temperature for 30 min. Later amonium phosphate was removed by centrifugation (69000 x g, 4 h) and the centrifugate was resuspendended in HEPES buffer (Roth, Germany), pH=7.4. LP presence was confirmed using HPTS pH indicator as described by Fritze et al. [5].

LP size was unified using Extruder system (Avanti Polar Lipids, USA). Susequently, LP size distribution was evaluated by dynamic light scattering method using Zetasizer Nano ZS (Malvern, Germany) system.

Freshly prepared LPs were incubated overnight with doxorubicin (Teva Pharma, Netherlands) at 4 °C. In order to eliminate free DOX, the repeated centrifugation was performed (69000 x g, 4 h). The concentration of DOX encapsulated in LPs was determined spectroscopically by evaluating the absorption at 499 nm (Beckman Coulter DU800, Brea, USA), after LPs were lysed using Triton X100 (Roth, Germany).

The subsequent experiments with CHO cells (1.3×10^6 cells/ml) were performed by adding Sonovue MBs (5.2×10^7 MBs/ml) and LPs loaded with DOX (at 10 μM final DOX concentration). The experimental groups were arranged: i) control (cells alone); ii) LP loaded DOX impact on cells (LP-DOX) group; iii) LP loaded DOX impact on cells with US irradiation (LP-DOX+US) group; iv) cavitation impact on cells (MB+US) group; v) LP loaded DOX impact on cells (LP-DOX+MB+US) group. During the insonication time (5 s), the cells were exposed to 1 MHz central frequency pulsed US at 10 % duty cycle (100 μs ON, 900 μs OFF) and 400 kPa acoustic peak negative pressure.

Cell viability was evaluated using cell clonogenic assay as it was described previously [4]. DOX delivery was evaluated by subtracting the mean of cell colony number in (LP-DOX+MB+US) group from (MB+US) group.

Results and discussion. The interval of LP size distribution was between 40-500 nm, with the major part of LPs (85 %) falling into 70-250 nm size range (Fig. 1).

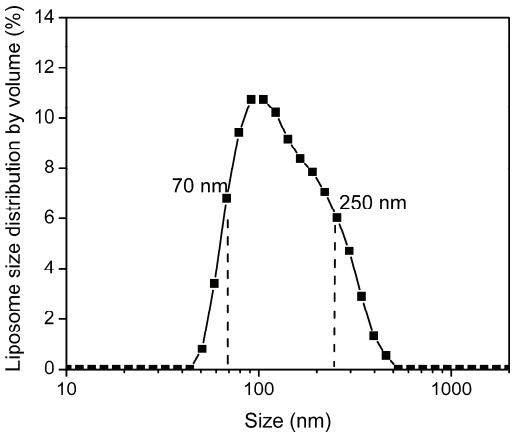

Fig. 1. LP size distribution

After LP loading with DOX, the amount of DOX in the LPs was estimated. Free DOX concentration was evaluated at 499 nm wavelength using standard calibration curve. DOX concentration dependence on optical density was evaluated to be linear in 1.83-183 μM concentration range. Fig. 2 (left) shows the absorption spectrum of liposome-released DOX (obtained after LP lysis). The corresponding concentration of liposome-released DOX was 13 μM corresponding to calibration curve (Fig. 2 right, insert).

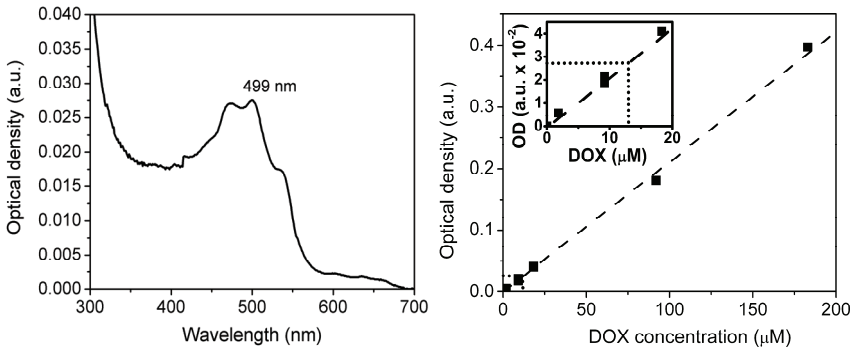

Fig. 2. DOX absorption of LP lysate (left) and the calibration curve of free DOX (right) used for determination of liposome-released DOX concentration (right, insert)

In this study the efficiency of DOX delivery into cells using LPs was evaluated by comparing DOX cytotoxicity within different experimental groups. The results show that DOX loaded LPs alone or administered with subsequent US application had only slight effect on cell viability decrease. Cavitation group (MB+US) and therapeutic (LP-DOX+MB+US) groups have produced significant cell viability decrease with final cell viability of ~ 40 and ~ 30 %, respectively. The cytotoxic effect of DOX was evaluated as the difference between the latter two groups and was estimated to be ~ 10 %.

The data obtained in this assay indicate that significant therapeutic effect is obtained only if DOX loaded LPs are used in tandem with MBs. This finding once again demonstrates that sonoporation efficiency is directly associated to MB cavitation and is line with our previous studies [3,4].

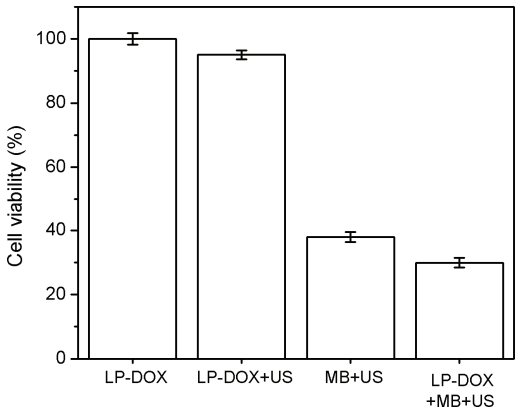

Fig. 3. The results of i*n vitro* experiments of DOX loaded LP delivery to cells

Conclusions. 1) DOX loaded LPs were successfully synthesized and used for cell viability diminishment *in vitro* 2) Pronounced DOX loaded LP therapeutic effect is induced by MB cavitation 3) The sonoporation experiments using DOX loaded LPs have shown promising results for the therapeutic applications.

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In the current study sonoporation was employed as a method for doxorubicin loaded liposome delivery to Chinese hamster ovary cells. The liposomes were successfully synthesized and loaded with doxorubicin to the therapeutic amount. The therapeutic effect of doxorubicin loaded liposomes combined with cell sonoporation resulted in 10 % cell viability decrease. The obtained results are promising as they indicate anticancer drug transfer opportunity to cells via sonoporation, simultaneously avoiding side-effects, common for *in vivo* applications.