Rapid Loading of Liposomes with Doxorubicin for Ovarian Cancer Treatment

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Background and Objectives

Ovarian cancer is the leading cause of death among gynecologic tumors [1]. Surgery plus platinum-based combination chemotherapy is the first-line management for the disease [2]. The treatment of patients with platinum-sensitive or platinum-resistant recurrent or refractory ovarian cancer can be further managed by employing liposomal Doxorubicin. The preparation of liposomes for cancer therapy is impeded by long-duration procedures that include drug loading at low temperature, extended purification steps by dialysis, waste of expensive drugs, generation of large amounts of hazardous waste, and other large associated costs. In this respect, our investigations focused on developing a rapid and effective procedure for loading the anticancer drug Doxorubicin into liposomes. To achieve these goals, we exploited the temperature-induced phase transition of lipids in combination with rapid purification of liposomes by high-speed centrifugation in an Airfuge; the combination enabled completion of liposome preparation and loading in one day.

Methodology

Principle of temperature-induced permeability. The key element of the proposed methodology is the phase transition of lipids assembled into artificial membrane systems (Figure 1). At high temperature, the lipids in a lipid membrane transition from a gel/solid crystalline phase to a more fluid phase, characterized by decreased rigidity and increased permeability. Therefore, we anticipated that an increased permeability will significantly speed up the active loading of drugs into liposomes.

Principle of active loading. Active loading exploits the molecules' ability to toggle between permeant and non-permeant state based on the solution conditions. In the case of the anticancer drug Dox and drug simulator AO, the state of the molecule depends on the solution's pH. At neutral pH, Dox and AO (weak bases, Figure 2) are electrically neutral and cross the membrane. However, at an acidic pH, the ionized Dox/AO may no longer cross the membrane and becomes trapped inside liposomes (Figure 2). Nonetheless, this process is very slow, and it takes several days at room or low temperature.

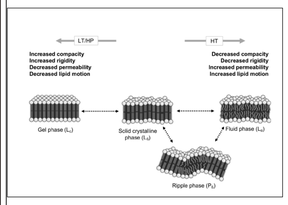

Figure 1. Thermal transition of lipids. Lipid disorganization leads to increased permeability

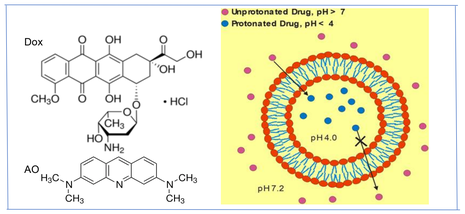

Figure 2. Principle of active loading. The chemical structure of Do and AO (left). The internal, acidic pH traps the ionized DOX inside liposomes (right).

Liposome preparation. To demonstrate the central hypothesis of this research project, we prepared by extrusion ~200 nm liposomes (therefore, capable of self accumulating into solid tumors) from lipid mixtures composed of DSPC, DSPE-PEG (to render them invisible to the immune host), and cholesterol. The liposomes were produced by hydrating the lipid cakes with a citrate buffer (pH 4.5). After preparation, the external buffer was exchanged with a neutral one (PBS1x, pH 7.2) by employing four consecutive purification steps in a high-speed Beckman Airfuge (75,000 RPM, 20 minutes). Liposome characterization was further performed by microscopy and Dynamic Light Scattering (DLS) analyses.

Liposomes loading at high temperature. After preparation, purification, and creation of a pH gradient, the liposomes have been exposed for one hour to 100 μM Dox or AO at 65 ^{0}C in a thermal block. After loading, the liposomes were cooled down by vial immersion in water at room temperature. The removal of the non-incorporated Dox or AO was performed by replacing the external neutral buffer with a drug-free solution (PBS 1x, pH 7.2) in four purification steps with the Airfuge.

Methodology (Cont.)

Loading verification. The presence of loaded Dox into liposomes was confirmed by fluorescence microscopy. Dox is a low-solubility anticancer drug, which presents fluorescence, thus it may be identified by microscopy. However, Dox also presents strong self-quenching capabilities (seen by a marked decrease in fluorescence as its concentration increases). AO, a drug simulator, is also fluorescent and presents self-quenching. This important feature can be exploited to additionally characterize the loading by monitoring the changes in fluorescence upon drug dilution into bulk after liposome membrane permeabilization with a non-ionic detergent (i.e., Triton X100).

Results and Discussion

Characterization of unloaded liposomes. Before loading, the undiluted liposomes were characterized by B/W microscopy and DLS (Figure 3). The image revealed the presence of high-density, non aggregated liposomes. DLS determination of size distribution indicated an average hydrodynamic diameter of 205 nm and an excellent Polydispersity Index (PDI) of 0.09, indicative of a very narrow size distribution. Liposomes of this size are suitable for self-accumulation into solid tumors by the Enhanced Permeability and Retention (EPR) effect, and the narrow distribution ensures their clinical applicability for drug delivery purposes.

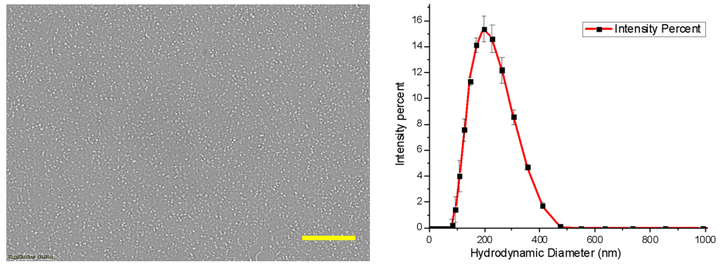

Figure 3 . Pre-loading characterization of liposomes. The unloaded liposomes have been characterized by B/W microscopy (left) and DLS (right) to determine the size distribution and assess potential aggregation before loading. The scale bar is 20 μm. The symbols in the DLS plot indicates mean values (n = 36) ± SD. The statistical analysis was carried out directly with the DLS software.

Characterization of Dox-loaded liposomes by microscopy and DLS. After sustained purification by high-speed centrifugation and washing, the loaded liposomes have been characterized for loading by fluorescence microscopy, DLS, and fluorescence spectroscopy. Fluorescence microscopy imaging of Dox-loaded liposomes was carried out on diluted samples (1:5) to avoid saturation elicited by the high density of liposomes in the samples and revealed the presence of loaded, non-aggregated liposomes (Figure 4). DLS indicated that the loading process did not affect the hydrodynamic size of the Dox loaded liposomes or the dispersity index.

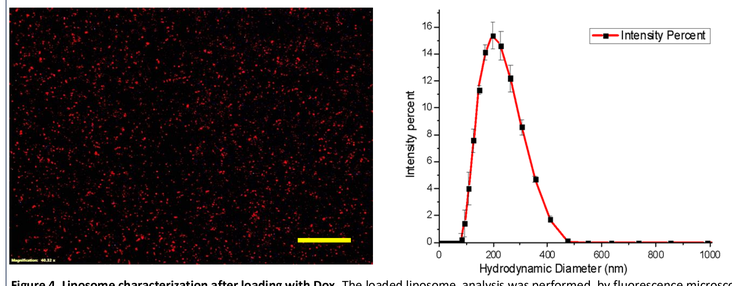

Figure 4. Liposome characterization after loading with Dox. The loaded liposome analysis was performed by fluorescence microscopy (left) and DLS (right), which indicate no change in size and aggregation status. The scale bar in the microscopy image is 20 μm. The symbols in the DLS plot indicates mean values (n = 36) ± SD. The statistical analysis was carried out directly with the DLS software.

Results and Discussion (Cont.)

Fluorescence spectroscopy analysis of Dox and AO release. Fluorescence spectroscopy analysis of Dox fluorescence dependency on concentration (Figure 5) shows a strong self-quenching effect (i.e. a marked decrease in fluorescence) at concentrations larger than ~30 μM; a similar plot is obtained for AO (data not shown). This self quenching feature is further exploited to assess the effectiveness of DOX and AO loading from the kinetic evolution of the fluorescence signal upon liposome breach by the detergent Triton X100. If the concentration of loaded Dox or AO exceeds the self quenching threshold, membrane solubilization leads to drug dilution into the bulk and an increase in fluorescence. This anticipated behavior was clearly observed in our experimental samples (Figure 5), indicative of significant Dox and AO loading into liposomes.

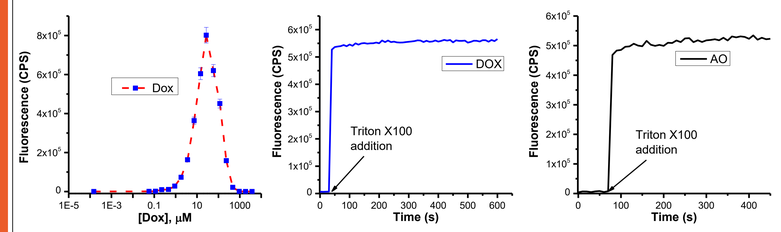

Figure 5. Fluorescence analysis of of loaded liposomes. Dox fluorescence indicates self-quenching at concentrations beyond 30 μM (left), Addition of Triton X100 to Dox-loaded liposomes leads to a substantial increase in fluorescence (middle), indicative of effective loading. Similarly, the increase in AO's fluorescence after detergent addition indicates successful active loading of the drug simulator (right). The symbol in the left plot indicates mean values (n = 3) ± SD.

Conclusion and Future Work

In conclusion, the thermal transition of lipids can be exploited to facilitate the rapid yet stable loading of liposomes with anticancer drugs and drug simulators by active approaches. In conjunction with fast separation by high-speed centrifugation, the entire loading and characterization procedure may be finalized in one day. This is a major advancement: the proposed procedure is substantially shorter than the traditional approach that implies dialysis and loading at low temperature, which requires at least one week. Future work will focus on protocol optimization for attaining a maximum load for various lipid compositions and distinct drugs. We are also planning on assessing the long-term stability of the thermally-loaded liposomes, together with their ability to self-accumulate into solid tumors.

Acknowledgements

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