Study on the *in situ* aggregation of liposomes with negatively charged phospholipids for use as injectable depot formulation

L. Rahnfeld[1], P. van Hoogevest[2], P. Luciani[1]

[1] Department of Pharmaceutical Technology, Friedrich Schiller University Jena, 07745 Jena, Germany
[2] Phospholipid Research Center, 69120 Heidelberg, Germany

Introduction

Compared to conventional parenteral formulations, injectable depot formulations owing to a sustained drug release offer several advantages, such as a reduced dosing frequency - and consequent improved compliance - or a predictable release profile. Additionally, fluctuations in the drug blood level could be smoothed and thereby side effects reduced [1].
Controlling the aggregation of negatively charged liposomal formulations could be used to induce a depot formation of a new injectable, long-acting drug delivery system. Although the aggregation behavior of negatively charged phospholipids (NCP) was already described [2,3], so far its use as depot formulation has not been investigated in a detailed manner.

Aims

Since phosphatidylserines (PS) were thoroughly investigated and the potential of phosphatidylglycerols (PG) and phosphatidic acids (PA) has not been fully exploited yet, the aim of this study was to screen different NCPs regarding their ability to form depots. Candidates with a noteworthy aggregation tendency were characterized further to evaluate the morphology and physicochemical properties of the aggregates and their potential as liposomal unit for depot formation in presence of divalent cations.

Methods

The different NCPs were mixed with the zwitterionic L-α-phosphatidylcholine from egg (EPC) or 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and for some formulations with cholesterol with a total lipid concentration of 5 mM. Large unilamellar vesicles were prepared by film hydration method, subjected to freeze-thaw-cycles and extruded through a 200 nm polycarbonate membrane. The mean hydrodynamic diameter and the size distribution (polydispersity index, PDI) of the liposomes were measured by dynamic light scattering. The zeta potential was determined by laser doppler micro-electrophoresis.
Liposome formulations were mixed with solutions of calcium chloride and magnesium chloride in a volume ratio of 1:3 and a cation concentration range from 0 mM to 10 mM. The optical density at 400 nm (OD_{400}) was determined.
Differential scanning calorimetry (DSC) was used for thermal behaviour investigations of the liposomes and aggregates. Multilamellar liposomes (50 mM) were mixed with different amounts of calcium chloride or magnesium chloride solutions.
The model drug bupivacaine hydrochloride (BUP) was encapsulated into liposome formulations (30 mM) using a remote loading method *via* the transmembrane ammonium gradient. The unencapsulated drug was removed by size exclusion chromatography and the encapsulated amount of BUP and the phospholipid concentration determined using HPLC.

Results

All tested liposome dispersions show a mean hydrodynamic diameter of around 150 nm and a low PDI, independently of the used NCPs. The zeta potential, indicating the surface charge of the vesicles with 25 mol% of NCP, was between -40 mV and -50 mV.
The extent of aggregation in presence of calcium or magnesium cations, assessed by turbidity measurement, suggested that the aggregation profile of the tested formulations is dependent on the nature of the phospholipid head group, the linked fatty acid and the used cation [4]. Particularly, EPC liposomes with 25 mol% DPPA, DPSG and DOPA in presence of either cation showed a desired aggregation profile. Formulations with 25 mol% NCP, 30 mol%

cholesterol and 45 mol% DPPC or EPC showed some differences in the aggregation profiles compared to the previous ones.
DSC studies showed differences between the two cations as well indicating differences in the binding to the PA or PG head groups. The sharp peaks of the phase transition of DPPA or DSPG liposomes were shifted to higher temperatures or disappeared upon addition of cations. First encapsulation experiments with the model drug BUP showed depending on the used NCP encapsulation efficiencies up to 60% with final drug-to-lipid ratios up to 0.1.

Conclusion
The three NCPs with the most pronounced aggregation behavior could be possible candidates for a depot formulation. The *in vitro* release profile of BUP from the aggregated liposomes is currently being investigated in order to proof the potential of the designed delivery system for a sustained drug release.

Keywords
Negatively charged phospholipid; Liposomes; Aggregation; Depot formulation

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