Liposome Encapsulating Estetrol for the Treatment of Ischemia Diseases in Premature Babies
C. Palazzo 1, R. Karim 1, T. Furst 1, C. Pequeux 1, E. Tskitishvili 1, M. Mawet 2, C. Maillard 2, J-M. Foidart 2, B. Evrard 1, G. Piel 1
1 Universite de Liege, 2 Estetra SPRL

Purpose
In 2010, almost 15 million of babies in the world are prematurely borned, 11.1 % of the total amount of alive children. Despite the better midwife and neonatology techniques, and the reduction in neonatology mortality, the number of babies with motor, vision, hearing or mental deficiencies is still constant along the last twenty years. Moreover, no efficacy treatment is available to the present day. The estetrol (E4) and its receptor ERα have an important role in the brain development. The E4 is involved in the proliferation, migration and differentiation of neuronal cells and it plays an important role in neuroprotection and anti-ischemic activity. The aim of this study is to develop a new liposome formulation encapsulating E4 in order to enhance its crossing of the blood-brain barrier (BBB). Moreover, to increase its low water solubility and its liposomal encapsulation, E4 has been complexed with cyclodextrins.

Methods
Preparation of E4-cyclodextrin (E4-CD) complex: Crysmeb® (CM) and Hydroxypropyl-β-cyclodextrin (degrees of substitution 0.87 and 0.63) (HPβCD 0.87 and HPβCD 0.63) water solutions were prepared at different concentrations. An excess of E4 was added to the cyclodextrin solutions and the dispersion was stirred at 140 rpm at 37°C for 24 hours. Insolubilized fraction of E4 was separated by filtration with a 0.2 µm mixed cellulose ester membrane.

Preparation of E4 loaded liposomes: Liposomes were prepared using the thin film hydration technique. The lipids 1,2-distearyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC) or 1-palmitoyl-2-oleoylphosphatidylcholine (POPC); dimethyldioctadecylammonium (DDAB) and cholesterol (1/0.6/0.2 molar ratio) were dissolved in ethanol. Ethanol was removed by rotary evaporation and the obtained lipid film was hydrated with HEPES buffer under vigorous stirring. In the case of E4 liposomes, E4 (20 % molar of the main lipid) was added with the lipids while for liposomes containing the E4-CD complex, the complex was used to rehydrate the lipid film instead of the buffer. The dispersion was extruded (400 nm, 200 nm and 100 nm filters). For the preparation of “stealth” liposomes, mPEG2000-DSPE (molar ratio 1/0.05 main lipid/ mPEG2000-DSPE) was added with post insertion-technique. Liposomes were purified by dialysis technique.

Results
Complexes of cyclodextrins CM and HPβCD with E4 were prepared, with a proportionally solubility increasing of the hormone. Due to the slightly lower solubility obtained with HPβCD ds 0.87, only HPβCD ds 0.63 was retained for future trials. The stability of the complexes, E4-CD (100 mM) was tested at different temperatures (4°C, 25°C and 37°C). The results obtained demonstrate that E4 complexed with Crysmeb® is unstable at the tested temperatures, while the complex HPβCD ds 0.63 - E4 is stable until 3 months. Liposomes encapsulating E4 with different phospholipids were prepared. All the formulations had an average particle size below 150 nm, polydispersion index below 0.100 and ζ potential around + 50 mV. Moreover, the encapsulation efficacy was between 3% and 10%. To increase the encapsulation efficacy, Drug-in-Cyclodextrin-in-Liposome (DCL) systems were developed. The formulations loaded with the complex HPβCD ds 0.63 - E4 showed a good encapsulation amount (between 15% and 35%) and kept the same size, ζ potential and polydispersion index as the classic formulations. To allow the intravenous administration, E4-liposomes and E4-DCLs were coated with mPEG2000. The resulting “stealth” liposomes had a lower ζ potential (around + 30 mV).

Conclusion
New liposome formulations containing E4 were prepared. DCL systems can be considered as a promising drug delivery system to target estrogens to the brain, due to their physiochemical characteristics.