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Effect of composition, hydrogenation of phospholipids and lyophilization on the characteristics of eugenol-loaded liposomes prepared by ethanol injection method

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ABSTRACT

The effect of composition, hydrogenation of phospholipids and lyophilization on liposomes characteristics was investigated. Liposomes were produced by ethanol injection method, employing hydrogenated (Phospholipon 80H, Phospholipon 90H) or non-hydrogenated soybean phosphatidylcholine (Lipoid S100) with cholesterol. Using Phospholipon 80H, cholesterol addition was crucial for liposomal homogeneity. Phospholipids concentration, stirring rate, and ethanol/water volume ratio were the most determinant factors controlling the vesicles size. Phospholipid assays showed that saturated phospholipids resulted in higher incorporation in vesicles than unsaturated ones. Smaller vesicles were obtained with Phospholipon 80H, due to lower content of phosphatidylcholine and to the presence of phosphatidylethanolamine which might increase repulsion between phospholipids, resulting in an increase in Zeta-potential and a decrease in vesicle size. TEM images revealed nanometric sized and spherical shaped oligo-lamellar vesicles. Although Lipoid S100 liposomes possessed the highest encapsulation efficiency of eugenol (86.6%), they were not stable in aqueous suspension at 4 °C after 2 months of storage, limiting their use in freeze-drying. Among several cryoprotectants, hydroxylpropyl-β-cyclodextrin protected eugenol-loaded Phospholipon 90H liposomes during freeze-drying as the mean vesicle size, polydispersity index, Zeta potential and encapsulation efficiency of eugenol were similar before and after lyophilization. This study demonstrated the possibility of maintaining a volatile essential oil in a suitable lyophilized liposomal formulation despite the very low applied pressures.

1. Introduction

Liposomes are intensively investigated and developed for the pharmaceutical, cosmetic, and food industries as carrier systems for the protection and delivery of bioactive agents. They are colloidal structures having an internal aqueous core formed by self-assembly of amphiphilic lipid molecules in solution. They are biodegradable, biocompatible, non-toxic, and non-immunogenic. They can carry hydrophilic substances in the aqueous internal cavity, hydrophobic compounds into the phospholipid membrane, and amphiphilic molecules at the water-bilayer interface (Laouini et al., 2012).

A liposomal formulation can be obtained by selecting an appropriate composition and production method. A major lipid membrane component is phosphatidylcholine. Phospholipids can be obtained from lecithin, a cheap and rich source of phosphatidylcholine. The cost of the formulations based on natural phospholipids, especially those derived from egg yolk or soybean phosphatidylcholine, is lower than that of liposomes made from synthetic phospholipids. Natural phospholipids can be modified by partial hydrogenation, leading to the formation of saturated phospholipids (Justo & Moraes, 2010).

Few studies compared hydrogenated or saturated liposomes to non-hydrogenated ones in terms of size, encapsulation efficiency (EE), stability, and biological activities. Trotta, Peira, Carlotti, and

Abbreviations: BPFs, bilayered phospholipids fragments; Chol, cholesterol; CD, cyclodextrin; DLS, dynamic light scattering; DMPC, dimyristoyl phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; DSPC, distearoyl phosphatidylcholine; EE, encapsulation efficiency; EPC, egg phosphatidylcholine, EPG, egg phosphatidylglycerol; Eug, eugenol; HP-β-CD, hydroxypropyl-beta-cyclodextrin; MLV, multilamellar vesicles; pdI, polydispersity index; PE, phosphatidylethanolamine; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; SPC, soybean phosphatidylcholine; SUV, small unilamellar vesicles.

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Gallarate (2004) produced deformable liposomes containing methotrexate by reverse-phase evaporation method using soybean lecithin or hydrogenated lecithin Phospholipon 100H and dipotassium glycyrrhizinate as surfactant. Their results showed that liposomes prepared using soybean lecithin (212 ± 25 nm) did not markedly differ in size from hydrogenated ones (235 ± 32 nm), whereas size increased with increasing the lipid/ dipotassium glycyrrhizinate ratio. Additionally, liposomes incorporating kanamycin prepared by ethanol injection method using unsaturated lipid EPC/Chol (60:40 mol%) possessed an average diameter of 116 ± 2.8 nm close to that formed of saturated lipids (60:40 mol%) like SPC/Chol (108 ± 3.3 nm), DSPC/Chol (108 ± 2.9 nm) and DPPC/Chol (136 ± 0.0 nm) (Justo & Moraes, 2005). However, using thin film hydration method, MLV empty liposomes prepared by hydrogenated Phospholipon 90H have a diameter of 327 ± 46 nm being smaller than non-hydrogenated P90 liposomes (457 nm ± 42). The same trend was observed with SUV empty liposomes, where P90H SUV had a diameter of 134 ± 21 nm versus 207 ± 30 nm for P90 SUV (Sinico et al., 2005).

With respect to EE, it has been demonstrated that hydrogenated phosphatidylcholine liposomes were optimal for the encapsulation of nisin Z (34.6%) versus unsaturated phospholipids vesicles (11.6%) (Laridi et al., 2003). In addition, the highest EE of kanamycin was achieved for saturated DSPC-based composition (57.8 ± 0.6%) compared to unsaturated EPC-based composition (11.3 ± 6.0%) (Justo et al., 2005). However, no significant differences concerning the EE of methotrexate was observed between soybean lecithin liposomes (31 ± 3%) and Phospholipon 100H (33 ± 2%) (Trotta et al., 2004). Similarly, a little difference in essential oil EE was obtained using either unsaturated or saturated phospholipids (74.1 ± 0.35% for P90 MLV versus 71.4 ± 0.58% for P90H MLV) (Sinico et al., 2005).

Besides, liposomes composed of unsaturated lipid EPC was far less stable than liposomes composed of fully saturated phospholipids like DSPC, DPPC or DMPC (Lian & Ho, 2001).

Concerning the biological activity, the effective concentration EC50 of *Artemisia arborescens* essential oil loaded liposomes against HSV-1 was found to be 43.6 and 18.3 mg/ml with P90 MLV and P90H MLV, respectively. This suggests that antiviral activity was enhanced with hydrogenated vesicles (Sinico et al., 2005).

On the other hand, several methods have been employed in lab-scale liposome preparation. The ethanol injection method developed by Batzri and Korn (1973) offers several advantages over the other ones used to prepare liposomes. It is simple, one step process, rapid, inexpensive, reproducible, avoids the use of hazardous solvents and strong forces that may disrupt the liposomes and the encapsulated molecules. This method produces small sized vesicles without any physical treatments such as sonication or extrusion and does not cause lipid degradation or oxidative alterations. This offers the possibility to use the ethanol injection technique for large-scale production (Batzri & Korn, 1973; Jaafar-Maalej, Diab, Andrieu, Elaissari, & Fessi, 2010; Justo & Moraes, 2010; Wagner, Platzgummer, & Kreismayr, 2006; Yang, Delaney, Ulrich, Schubert, & Fahr, 2012). This method is suitable for the entrapment of hydrophobic, hydrophilic (Jaafar-Maalej et al., 2010), and amphiphilic molecules (Wagner et al., 2006).

Recently, essential oils have gained increasing interest in food chemistry and pharmaceutical industries, owing a wide range of biological activities. Eugenol (4-allyl-2-methoxyphenol; Eug) (Fig. 1), approved by the Food and Drug Administration, has been extensively studied for its antibacterial (Catherine, Deepika, & Negi, 2012), antifungal (Vazquez, Fente, Franco, Vazquez, & Cepeda, 2001), and antioxidant (Sebaaly, Jraij, Fessi, Charcosset, & Greige-Gerges, 2015) effects. However, light sensitivity, volatility and poor water solubility of Eug limits its large application. Therefore, the encapsulation technique would be an appropriate method to circumvent these drawbacks.

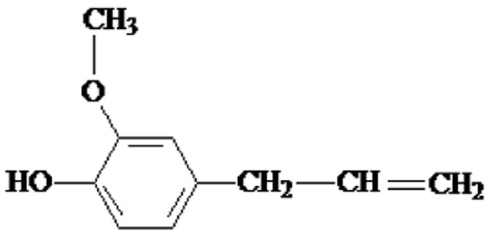

Fig. 1. Chemical structure of eugenol.

Liposomes can be stored either on freeze-dried state or as an aqueous dispersion. As an aqueous dispersion, their physical and chemical stabilities such as leakage of encapsulated drugs, liposomes aggregation, hydrolysis, and oxidation of phospholipids constitute major limitation for long-term stability (Chen, Han, Cai, & Tang, 2010). Lyophilization is the main approach used to overcome these problems and it is widely applicable in the pharmaceutical and food industries (Chen et al., 2010). However, it may lead to membrane damage of liposomes. So to maintain the same particle size distribution and to avoid leakage of the encapsulated drug from liposomes during lyophilization, a suitable cryoprotectant needs to be added (Janicki, Jankowski, Szulc, Woyczikowski, & Sznitowska, 2002).

The present study is dedicated to the development of an appropriate liposomal formulation using cheap soybean phospholipids to preserve essential oil components and eugenol is used as a model. The effect of composition and hydrogenation of soybean phospholipids on the characteristics of liposomes prepared by ethanol injection method was investigated. To get an optimized formulation, the effect of several parameters on both liposomes size and pdI was studied using Phospholipon 80H as a phospholipid model. Based on the optimal conditions, liposome batches were prepared, in the absence and presence of eugenol, employing either hydrogenated (Phospholipon 80H, Phospholipon 9H) or non-hydrogenated soybean phosphatidylcholine (Lipoid S100) with cholesterol. Phospholipid loading rate was determined. Liposomes were characterized in terms of size, pdI, Zeta potential, morphology, and EE of Eug. A stability study was then performed at 4 °C for the various batches. Finally, the effect of various cryoprotectants on the properties of Eug-loaded phospholipon 90H liposomes obtained after freeze-drying was investigated.

2. Materials and methods

2.1. Materials

Hydrogenated Phospholipon 80H (78.9% soybean phosphatidylcholine, 3.9% lysophosphatidylcholine, 0.5% water, 0.1% ethanol and the rest is phosphatidylethanolamine (PE) and some lysophosphatidylethanolamine, little amount of triglyceride) or hydrogenated Phospholipon 90H (90% soybean phosphatidylcholine, 4% lysophosphatidylcholine, 2% triglycerides, 2% water, 0.5% ethanol, iodine 1%) and non-hydrogenated soy phosphatidylcholine Lipoid S100 (94% soybean phosphatidylcholine, 3% lysophosphatidylcholine, 0.5% N-acyl-phosphatidylethanolamine, 0.1% phosphatidylethanolamine, 0.1% phosphatidylinositol, 2% water, 0.2% ethanol) were supplied by Lipoid GmbH (Germany). Absolute ethanol, eugenol, thymol, cholesterol, ammonium molybdate, hydrogen peroxide, potassium dihydrogenophosphate, methanol-HPLC grade, cetyl alcohol, sodium silicotungstate, and 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) were purchased from Sigma-Aldrich (France). Sulfuric acid was purchased from ACROS organics (New Jersey, USA) and 4-amino-3-hydroxy-1-naphthalene sulfonic