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PREPARATION AND CHARACTERIZATION OF LIPOSOMES LOADED WITH POLYPHENOLS EXTRACTED FROM CALLENDULAE FLOS

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Abstract

Liposomes have been used since 1960 in various fields such as: chemistry, mathematics, physics, biophysics, and biology. Since then they are successfully available in modern technology as an active transport molecule at the site of action. They are spherical vesicles, made up of one, or more, bilateral phospholipid layers. Phospholipids are the main component of the cell membrane, containing a hydrophilic and a hydrophobic part.

They are most often used as carriers for a large number of molecules. The cosmetic, pharmaceutical, agriculture and food industries have studied the use of liposome encapsulation to streamline the transport and delivery of different systems that can trap unstable components (flavors, antimicrobial substances, antioxidants, and bioactive elements) and make them more functional.

Calendula officinalis L. *is used all around the world in the pharmaceutic and cosmetic industry for its antioxidant, antibacterial, antifungal, anti-inflammatory, and antiviral properties. For external use, it is useful for treating skin inflammations, scratches, wound healing and burns.*

At present, liposomes are widely used in the medical field. They are considered the most suitable carriers for the introduction and transport to the target organ of various agents, for example anticancer, anti-inflammatory, antibiotic, hormonal, antifungal, enzymatic, vaccines (in the aqueous or lipid phases). The use of different liposomes dimensions and compositions is done to ensure adequate time of delivery.

Key words: Liposomes; phospholipids; wound healing; *Calendula officinalis* L.; burns

INTRODUCTION

Polyphenols are bioactive compounds that have beneficial effects, being used mainly in the health, cosmetic and food industries. After being extracted from medicinal plants, they have several disadvantages: low water solubility, low physico-chemical stability, which leads to reduced bioavailability, bitter and astringent taste. In the present study, we encapsulated polyphenols from *Calendula officinalis* L. flowers extract into nanostructured liposomal systems to improve their characteristics.

Liposomes are small, artificial, spherical vesicles that can be created from natural, non-toxic cholesterol and phospholipids (Akbarzadeh A. et al.,

2013). Due to their low toxicity, biocompatibility and ability to trap both lipophilic and hydrophilic substances, liposomes are seen as drug delivery systems. The structure, chemical composition and size of liposomes can be controlled by the preparation method (Ahmed K.S. et al., 2018).

When phospholipids are dispersed in aqueous solutions, they have a strong tendency to form membranes and this is due to the amphipathic nature (Papahadjopoulos D, Kimelberg H.K., 1974).

Liposomes can have single or double lipid membranes, and their size can range from very small vesicles (0.025 μm) to large vesicles (2.5 μm). Both the size and the number of bilateral layers influence the amount of drug that has been incorporated into liposomes.

Compared to the micrometer-size carriers that are obtained by traditional techniques, nanoparticles have the advantage of having a larger interfacial surface, increase solubility and improve bioavailability, taste, aroma of bioactive compounds and the active principle released in a controlled manner (Singh H., 2016; Bochicchio S. et al., 2016; (Putheti S., 2015). There are two categories of liposomes, classified according to the size of the vesicle and the double lipid layers: multilamellar vesicles (> 500 nm; MLV) or unilamellar (> 100nm; LUV), unilamellar ones can be found in 3 forms: small unilamellar vesicles (20 -100 nm called SUV), intermediate (100-250 nm), or large unilamellar vesicles (> 250 nm, called LUV) (Pattni B.S. et al., 2015); (Jesorka A., Orwar O., 2008; Makino K., Shibata A., 2006; Irache J.M. et al., 2001).

When choosing the method of liposome preparation, certain factors are taken into account: the physico-chemical characteristics of the components of the liposome and the drug to be loaded, the toxicity and concentration of the substance to be loaded, the environment in which the liposomes will be dispersed, the additional processes what will occur during liposome administration, the size and half-life that is desired to be achieved, the costs and the large scale applicability for the clinical purpose (Bozzuto G., Molinari A., 2015; Gomez-Hens A, Fernandez-Romero J.M, 2006; Mozafari M.R. et al., 2008; Dua J.S. et al., 2012; Cagdas M. et al., 2014).

Due to its simplicity and low costs, many laboratories use the method of obtaining liposomes by hydrating the lipid film, in which the lipid components are dissolved in an organic solvent (Bulbake U. et al., 2017). This involves drying a lipid solution, forming a thin film at the base of the round bottom flask and then hydrating the film with an aqueous buffer, shaking for a certain period of time. The components to be encapsulated will be added in an organic solvent or aqueous medium, choosing the method based on their solubility.

To evaluate the liposomes size distribution, various techniques are used such as: microscopy, size exclusion chromatography (SEC), field flow

fractionation or even static or dynamic light scattering (Nogueira E. et al., 2015). The most direct method for determining the size of the liposome is performed with the help of an electron microscope that offers the possibility of visualizing the liposomes individually (Ho R.J.J. et al., 1986).

MATERIAL AND METHOD

Materials: The liposomes loaded with polyphenols from the *Calendula officinalis* L. flowers extract were prepared by the hydration method from phosphatidylcholine, dipalmitoyl phosphatidylcholine, cholesterol purchased from Sigma-Aldrich (Milan. Italy). We used flowers of *Calendula officinalis* L. harvested from the Crișana region, Bihor County. The compounds used are of adequate purity, attested by analysis bulletins issued by the manufacturer.

Preparation of liposomes

When preparing liposomes with different tips of lipids, those one must first be dissolved and mixed in an organic solvent to assure a homogeneous mixture of lipids. Most times, this process is carried out using chloroform to obtain a clear lipid solution for complete mixing of lipids. Once the lipids are mixed in the chloroform, the solvent is removed to yield a lipid film. For larger volumes, the organic solvent should be removed by rotary evaporation until a thin lipid film is present on the sides of a round bottom flask. The lipid film is truly dried and without residual organic solvent by placing the flask on a vacuum pump. Spinning the round bottom flask in the warm water bath maintained at a temperature of 40°C, allows the lipid to hydrate in its fluid phase with adequate agitation.

Hydration of the dry lipid film is achieved by adding an aqueous medium to the container of dry lipid and then agitating. The application of the lipid vesicles is generally determined by the hydration medium which can include distilled water, buffer solutions, saline, and non-electrolytes such as sugar solutions. To develop liposome vesicular systems, we used flowers of Calendula officinalis *L*. The flowers were carefully selected as to obtain an alcoholic extract by maceration with 30% (v/v) alcohol, plant-to-solvent ratio 1:5.

The liposomes were prepared from phosphatidylcholine (50 mg), dipalmitoyl phosphatidylcholine (50 mg), cholesterol (2,5 mg). The method chosen for preparation was the thin-film. This method produces micrometric structures, with an ultrasound-assisted process developed to prepare nanometric structures. The desired size of liposomes, is determinate during the production process, decreasing due to addition of ultrasound energy,

used to break the lipid bilayer into small pieces that close themselves in spherical structures.

At first lipids were dissolved in organic solvent (2 ml of chloroform), after mixing, the solution was transferred to a rotary evaporation, at 40°C, 80 rpm and 200 mBars the solvents were removed and the solution was vacuum-dried. A yellow film was formed on the sides of a round bottom flask. The dried lipid film, was hydrated at room temperature with 2 ml solution of phosphate buffer (0,25 M, at pH 6,5), and then mixed in a centrifuge at 60°C, 200 rpm for 20 minutes. After that, the suspension was homogenized through an ultrasound-assist process (25°C, for 30 minutes) leading to the nanoliposome formation. We used the ultrasound-assist process in nanoliposomes production, because the energy is used to break the lipid bilayer into small pieces which closes themselves in spherical structures producing SUVs. Finally, the suspension was recovered and then characterized.

DLS (Dynamic Light Scattering) and ZP (zeta potential) measurements were carried out to determinate the physicochemical and morphological properties of the nanoparticles.

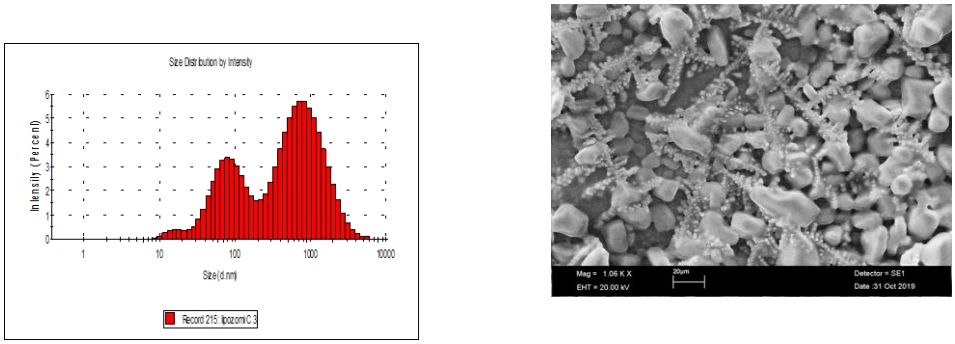
Another method for the size determination on liposomes is scanning electron microscope (SEM) which produces images of a sample by scanning the surface with a focused beam of electrons. SEM can achieve resolution better than 1 nanometer.

Also, we used Scanning Electron Microscope (SEM, LEO 435 VP) which allows the visualization of dynamic changes that occur to vesicle as physical environmental parameters are changed. The characteristics of Scanning Electron Microscope used: Vacuum 2.17e-005 mBar, Detector SE1, Beam current 250uA, I Probe 200pA, Filament I Target aprox 2.180 A, EHT Target 20kV, Enlarge 1.06 KX, Reference scale 20.00 kV.

The entrapment efficiency was determined immediately after preparation, after 24 hours, 1 week, 2 weeks, 30 days and 60 days. The Folin-Ciocâlteu test using gallic acid as a standard was used for evaluation. A testing system of six Franz diffusion cells and the UV spectrophotometric method were used to determinate the release of the polyphenolic complex from the liposomal nanoparticles.

RESULTS AND DISCUSSION

The size and the polydispersity index of the liposomes was made at 25°C, and after ultrafiltration (Pellicon® 2 Cassette Ultrafiltration) we separated the particles with nanometric dimensions smaller than 500 nm (Fig.1.).



(a) (b)

Fig.1. DLS analysis of liposomal systems:
a) Particle size distribution; b) Zeta potential measurement

The obtained liposomal systems showed high entrapment efficiency of 81.02±0.42% and stability at 25°C for at least 60 days. Several kinetic models (order zero, order I, Higuchi) were used to investigate the mechanism of in vitro release of polyphenols. All the entrapped polyphenols were released in 24 hours (98.15±0.22%).

CONCLUSIONS

All the achieved positive results endorse the use of pharmaceutical applications for nanoliposomes loaded with *Calendula officinalis* flowers because the evaluation demonstrated good entrapment efficiency (~80%), small size (less than 500 nm), low polydispersity index and good stability after 60 days at 25°C, avoiding the use of toxic solvents and drastic conditions.

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