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Incorporation of Biologically Active Ingredient Gallic Acid into Nano-scale Lipid Vesicles

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Nano-scale lipid vesicles or liposomes are extremely important lipid particles because of their unique properties and possible incorporation of various biologically active substances into their interior. Therefore, they can be used for numerous biomedical applications. Liposomes have the ability to protect incorporated bioactive substances, thereby preserving their function. They have a significant advantage over various nanoparticles, as they can bind and transfer hydrophobic, hydrophilic, and amphiphilic compounds.
The aim of the study was the synthesis of liposomes, suitable for the potential encapsulation of active ingredients for pharmaceutical and clinical purposes. Liposomes were prepared using a thin lipid film hydration method with glass beads. The synthesized liposomes were characterized by measuring the zeta potential to determine their stability, polydispersity index, and particle size. Furthermore, the biologically active ingredient gallic acid (GA) was incorporated into the lipid vesicles at different concentrations. The encapsulation efficiency of the active ingredient GA in liposomes and the *in vitro* release of the encapsulated bioactive component were studied using the dialysis technique. Synthesized nano-scale lipid vesicles were found to be stable, with an average size of 181.5 nm. The highest encapsulation efficiency (98.3%) and the highest percentage of released bioactive substance (38.3%) were obtained at 0.1 mg/mL of GA. GA-incorporated nano-scale lipid vesicles are promising as suitable carriers of bioactive ingredients for various therapeutic purposes.

1. Introduction

Liposomes are self-assembled spherical, nano-scale lipid vesicles consisting of one or more concentric phospholipid bilayers surrounding a hydrophilic core (Huang et al., 2022). They have the unique ability to successfully load molecules with different solubilities, namely hydrophobic, hydrophilic, and amphiphilic molecules. For this reason, these nano-scale vesicles can be used as delivery systems for a variety of drugs. Hydrophobic molecules can be loaded into the lipid bilayer membrane, hydrophilic molecules into the water center. While amphiphilic molecules can be entrapped at the water/lipid interface (Figure 1) (Sercombe et al., 2015).
Liposomes have emerged as promising delivery systems for various therapeutic agents, suitable for numerous biomedical applications. They allow the stabilization of encapsulated drugs, which can be successfully protected from enzymatic degradation, chemical and immunological inactivation, and rapid plasma clearance, which significantly contributes to the improvement and prolongation of therapeutic effect (Guimarães et al., 2021). Therefore, liposomes also offer the possibility of prolonging the half-life of the drug (Hemetsberger et al., 2022). In addition, they enable a significant improvement in the biodistribution of therapeutic agents at the selected target site (Guimarães et al., 2021). Moreover, their remarkable properties, such as biocompatibility, biodegradability, high versatility, non-toxicity, non-immunogenicity, and non-pathogenicity, make them one of the most promising drug delivery systems (Cheng et al., 2020).
Liposomes can be prepared from natural phospholipids, the composition of which has a significant influence on the properties of the synthesized liposomes, such as particle size, fluidity, rigidity, electrical charge, and stability (Nsairat et al., 2022). The properties of liposomes are also strongly influenced by the synthesis technique (Lombardo and Kiselev, 2022). Liposomes prepared from unsaturated phosphatidylcholine species from natural

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sources, such as egg and soy phosphatidylcholine, are highly permeable and less stable. On the other hand, if phospholipids saturated with lipids with long acyl chains are used, such as dipalmitoylphosphatidylcholine, the liposomes produced have rigid and nearly impermeable bilayer structures (Leitgeb et al., 2020). Based on their size and lamellarity, liposomes can be divided into small unilamellar vesicles (SUV, size range 20-100 nm), large unilamellar vesicles (LUV, size >100 nm), giant unilamellar vesicles (GUV, size >1000 nm), oligolamellar vesicles (OLV, size range 100-1000 nm), multilamellar vesicles (MLV, >500 nm), and multivesicular vesicles (MVV, >1000 nm) (Isalomboto Nkanga et al., 2019).

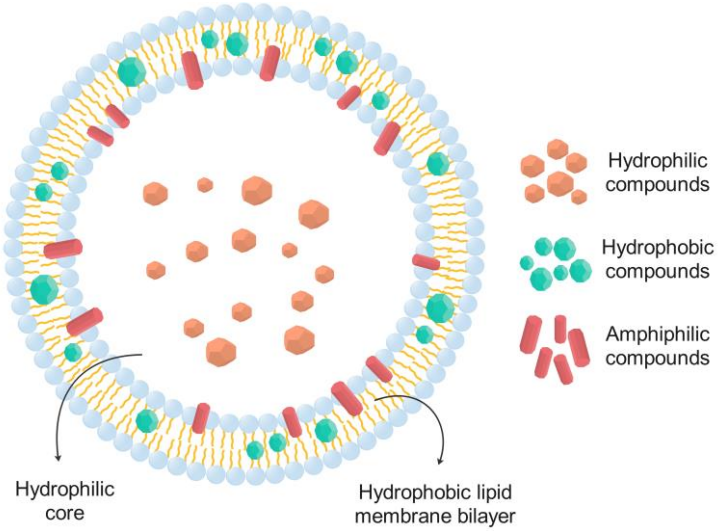

Figure 1: General structure of liposomes and possible incorporation of hydrophilic, hydrophobic, and amphiphilic compounds.

Various methods have been developed for the successful synthesis of liposomes, namely mechanical and solvent dispersion methods (Tagrida et al., 2021). Mechanical dispersion methods include the thin lipid film hydration, the French press cell, the freeze-thaw, sonication, microemulsification, and membrane extrusion. Solvent dispersion methods include reverse-phase evaporation, ethanol injection, ether injection (solvent vaporization), and double emulsification (Akbarzadeh et al., 2013). The choice of an appropriate method for the synthesis of liposomes depends on several factors, in particular, the physicochemical properties, the concentration and toxicity of the incorporated therapeutic substance, the type of solution used to disperse the liposomes, the size of the liposomes, the polydispersity of the liposomes, the synthesis cost, the encapsulation efficiency, and the sustained release at the target site (Tomnikova et al., 2022). The most commonly used technique for liposome preparation is the thin lipid film hydration method (Šturm and Poklar Ulrih, 2021). It is based on the use of an organic solvent to prepare a lipid solution, followed by evaporation of the solvent under reduced pressure, hydration, filtration, and purification of the product (Wang et al., 2017).
Liposomes are used to encapsulate unstable compounds such as antioxidants, antimicrobial compounds, bioactive elements, and antigenic proteins that play an essential role in human health. Encapsulation in liposomes successfully preserves their functionality (Simão et al., 2015). Furthermore, biologically active substances may be degraded in the gastrointestinal tract, resulting in an insufficient therapeutic effect (Păvăloiu et al., 2021). Therefore, liposomes loaded with the hydrophilic bioactive ingredient gallic acid (GA) were prepared using an inexpensive and practical laboratory procedure, a thin lipid film hydration method with glass beads. The liposomal formulation was studied in terms of its stability, particle size, and size distribution. The encapsulation efficiency and *in vitro* release study of the encapsulated GA were also investigated.

2. Materials and methods

2.1 Preparation of gallic acid-loaded liposomes

Liposome formulation was performed using a thin lipid film hydration method with glass beads. Soy phosphatidylcholine and cholesterol were dissolved in a ratio of 3:1 (w/w) in the organic solvent ethanol with constant stirring and heating to 50 °C until complete dissolution. Ethanol was then completely removed under reduced pressure using a rotary evaporator, resulting in the formation of a thin lipid film on the wall of the flask. The resulting film was then hydrated with a solution of GA in various concentrations, and an appropriate amount

of glass beads with a diameter of 5 mm was added. The flask was then shaken on a shaker at 200 rpm and 20 °C for 24 hours. Figure 2 shows the process for the preparation of liposomes loaded with GA.

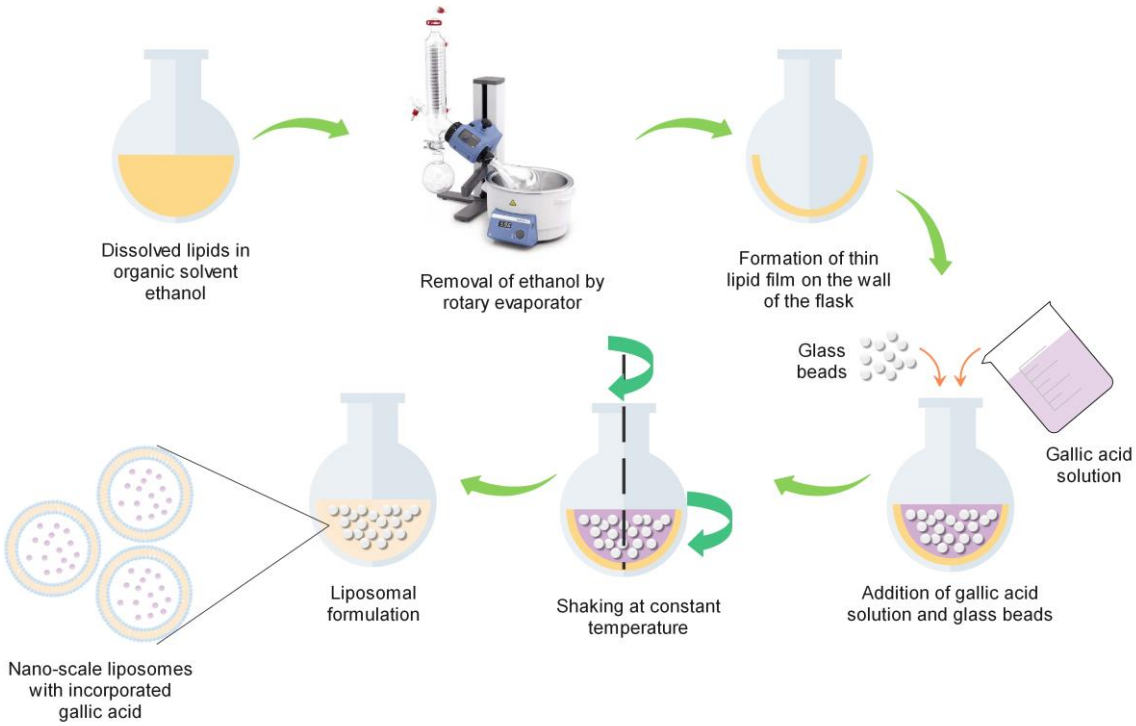

Figure 2: Schematic representation of the preparation of GA-loaded liposomes using a thin lipid film hydration method with glass beads.

2.2 Characterization of liposomes

Liposomes were characterized by measuring the mean particle size, size distribution expressed as polydispersity index (PDI), and zeta potential by determining the surface charge of liposomes to determine their stability by the dynamic light scattering (DLS) analysis using Zeta sizer Nano ZS instrument at room temperature.

2.3 Encapsulation efficiency of bioactive ingredient

GA was incorporated in liposomes at three different concentrations, 0.05, 0.1, and 0.5 mg/ml, prepared in 0.01 M PBS buffer at pH 5. The encapsulation efficiency of GA in liposomes was determined by the dialysis technique. 1 ml of the synthesized liposomes with incorporated GA was transferred to a dialysis bag (cut off 8000-12000), which was then immersed in 25 ml of PBS buffer. Dialysis was performed using a magnetic stirrer at 100 rpm and room temperature. Samples were analyzed by the Folin-Ciocalteu spectrophotometric method to determine total phenolic content. Encapsulation efficiency (%) was calculated using Equation 1:

$$EE = \frac{\gamma_{total\,GA} - \gamma_{free\,GA}}{\gamma_{total\,GA}} \cdot 100\% \quad (1)$$

Where is:
EE - encapsulation efficiency (%) of GA into liposomes,
$\gamma_{total\,GA}$ – concentration of total GA in liposomes (mg/ml),
$\gamma_{free\,GA}$ – concentration of free GA (mg/ml).

2.4 *In vitro* release kinetics of encapsulated bioactive ingredient

The kinetics of the release of GA from liposomes at body temperature was studied. 5 ml of the synthesized liposomes with incorporated GA at different concentrations were transferred to a dialysis bag (cut off 8000-12000) and immersed in 25 ml of PBS. This was followed by incubation at 37 °C with constant shaking at 100 rpm. Samples of 0.5 ml were taken at various time intervals, and the dialysate was replaced with an equal volume of fresh PBS buffer. The samples were analyzed by the Folin-Ciocalteu spectrophotometric method to

determine the total phenolic content. The cumulative release (%) of GA from liposomes was calculated using Equation 2.

$$CR = \frac{M_{GA,t}}{M_{GA,\infty}} \cdot 100\% \quad (2)$$

Where is:
CR – cumulative release of GA (%),
$M_{GA,t}$ – the amount of total GA released from liposomes at different time intervals (mg/ml),
$M_{GA,\infty}$ – the amount of GA loaded into liposomes (mg/ml).

3. Results and discussion

3.1 Characterization of liposomes

Liposomes were successfully synthesized by the thin lipid film hydration method using 5 mm glass beads with shaking for 24 hours. The DLS technique was used to determine the mean particle size, PDI, and zeta potential. The results show that large unilamellar vesicles (>100 nm) were synthesized, as an average liposome vesicle diameter of 181.5 nm was determined. The desirable particle size for drug delivery is between 50 and 200 nm. Therefore, the nano-scale lipid vesicles produced in our study are suitable for drug delivery applications. The PDI value is a measure of the heterogeneity of the sample with respect to particle size. Based on the PDI value determined, which was less than 0.3, it can be confirmed that the synthesized liposomes were uniform or homogeneous. Zeta potential is a parameter that indicates how stable the liposomal formulation is. Liposomes with a zeta potential greater than 30 mV are considered stable, regardless of the charge. The measured zeta potential of the synthesized liposomes was negative, indicating a negative charge on their surface. A value of -53 mV was determined, confirming a stable liposomal formulation.

3.2 Encapsulation efficiency of bioactive ingredient

Hydrophilic bioactive ingredient GA was successfully incorporated into liposomes at three different concentrations. Encapsulation efficiency was determined using the dialysis technique. Figure 3 shows the obtained encapsulation efficiencies for all three tested concentrations of GA in the liposomes.

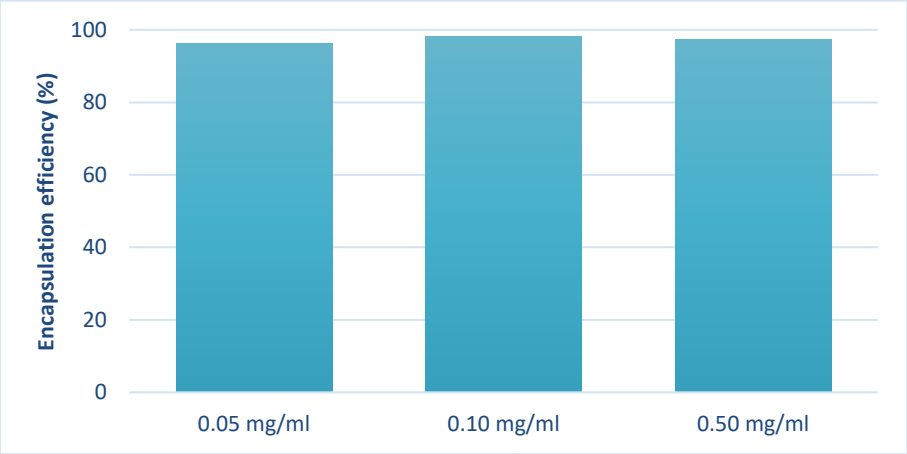

Figure 3: Encapsulation efficiency of GA in liposomes at three different concentrations.

The obtained percentages of encapsulation efficiency of three different concentrations of GA in liposomes are comparable, as they do not differ by less than 3%. However, the highest encapsulation efficiency (98.3%) was obtained with GA at a 0.1 mg/ml concentration.

3.3 *In vitro* release study of encapsulated bioactive ingredient

For the synthesized liposomes with incorporated GA at three different concentrations (0.5, 0.10, and 0.50 mg/ml), the percentage of GA released from the liposomes was determined after exposure at 37 °C for 24 hours and shaking at 100 rpm. Figure 4 shows the percentage of GA released after 24 hours for all three concentrations.

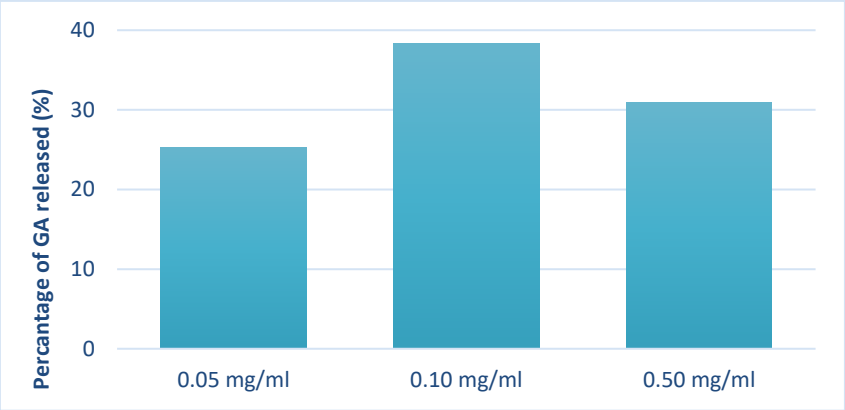

Figure 4: Percentage of GA released from liposomes after 24 hours at 37 °C.

The maximum percentage of *in vitro* release after 24-hour exposure of liposomes to PBS at body temperature (37 °C) was reached at a GA concentration of 0.1 mg/ml (38.3%). Therefore, among the tested concentrations of GA, 0.1 mg/ml was the concentration where the highest release rate of the tested bioactive ingredient from liposomes was detected. Furthermore, the kinetics of *in vitro* release of GA from liposomes at different concentrations of GA encapsulated in the liposomes was studied. The obtained results are shown in Figure 5.

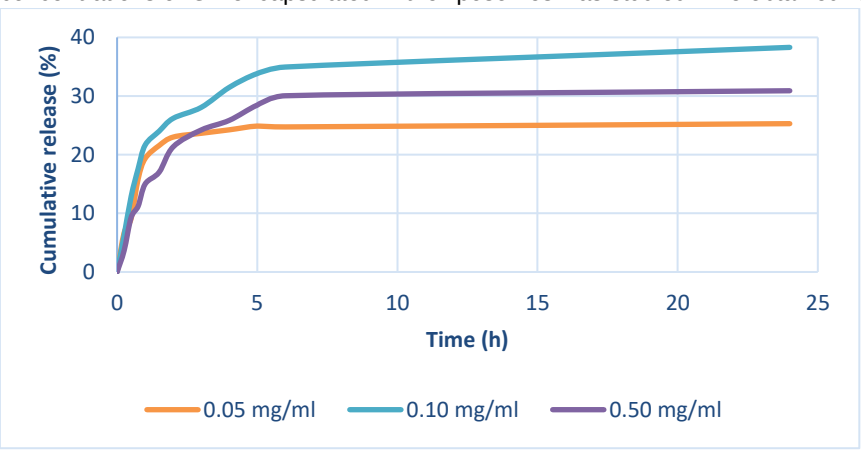

Figure 5: Kinetics of in vitro release of GA from liposomes at 37 °C.

In an *in vitro* study of the release of GA from liposomes, it was found that the release initially increased steeply at all tested concentrations. After 6 h, no significant differences in released GA were observed with further increase in release time. The most efficient GA release was achieved when 0.1 mg/ml of GA was incorporated into liposomes. At lower and higher GA concentrations tested, the release was less efficient.

4. Conclusions

Liposomes are important nano-scale lipid vesicles for the potential encapsulation of bioactive ingredients, especially for pharmaceutical, cosmetics, food, and agriculture purposes. Therefore, we have focused on using an organic solvent, ethanol, which is non-toxic and harmless to humans and nature. Liposomes were prepared using a thin lipid film hydration method with glass beads. The synthesized liposomes with uniform size distribution (PDI<3) had an average diameter of 181.5 nm, a desirable particle size for drug delivery applications. The zeta potential value was -53 mV, indicating a stable formation of nano-lipid vesicles. Furthermore, we have successfully incorporated the active ingredient GA (EE = 98.3%) into liposomes. Namely, GA is extremely important for human health and is frequently used as an additive in the pharmaceutical and food industries. In studying the kinetics of the *in vitro* release of GA from liposomes, we found that the release from liposomes increased steeply at the beginning. Later (after 6 hours), no significant differences in the released GA were observed with increasing release time. The highest encapsulation efficiency and percentage of the release of the active ingredient were achieved at a concentration of incorporated GA of 0.1 mg/ml. Therefore, nano-scale

lipid vesicles loaded with bioactive compounds such as GA are promising as suitable nanocarriers for various biomedical applications to achieve sustained release of the incorporated bioactive ingredient.

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