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Faculty of Pharmacy

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Summary of Ph.D. thesis

QBD APPROACH FOR OPTIMIZATION AND CHARACTERIZATION OF ANTI-GLIOBLASTOMA DRUG EMBEDDED LIPID NANOPARTICLES FOR INTRANASAL ADMINISTRATION

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1. INTRODUCTION

Glioblastoma multiforme (GBM) is the most aggressive type of life-threatening glial brain tumor and have poor diagnosis [1-3]. The nose to brain delivery is simple and direct approach including many advantages of enhanced bioavailability, shorter onset of action, and non-invasiveness [4-5]. There are many risk factors that should be taken into account, including particle characteristics, dissolution, structure and permeability profiles and the production method as well. Therefore, QbD approach was applied for the quality management based on required quality to fulfill the therapeutic need. [6, 7]. Lipid nanoparticles (liposomes, SLNs) were developed and hyaluronic acid (HA) was applied to increase the muco-adhesivity, as an essential strategy for intranasal drug delivery. [8].

A novel direct pouring method (DPM) a modified form of solvent emulsification and injection method was used for the development of coated liposomes [12]. Our study utilized two drugs n-propylgallate (PG) (propyl 3,4,5-tri-hydroxybenzoate) and lomustine (LOM) (a nitrosourea compound and a chemotherapeutic agent) [13]. Different lipid nano-formulations were formulated, namely HA-coated liposomes loaded with PG, PG-SLNs [14], LOM liposomes and PG-LOM liposomes. These lipid nano-formulations were characterized and investigated for its suitability via intranasal administration. The anti-proliferative effect of optimized formulations was studied and compared on different types of cancer cells lines [15].

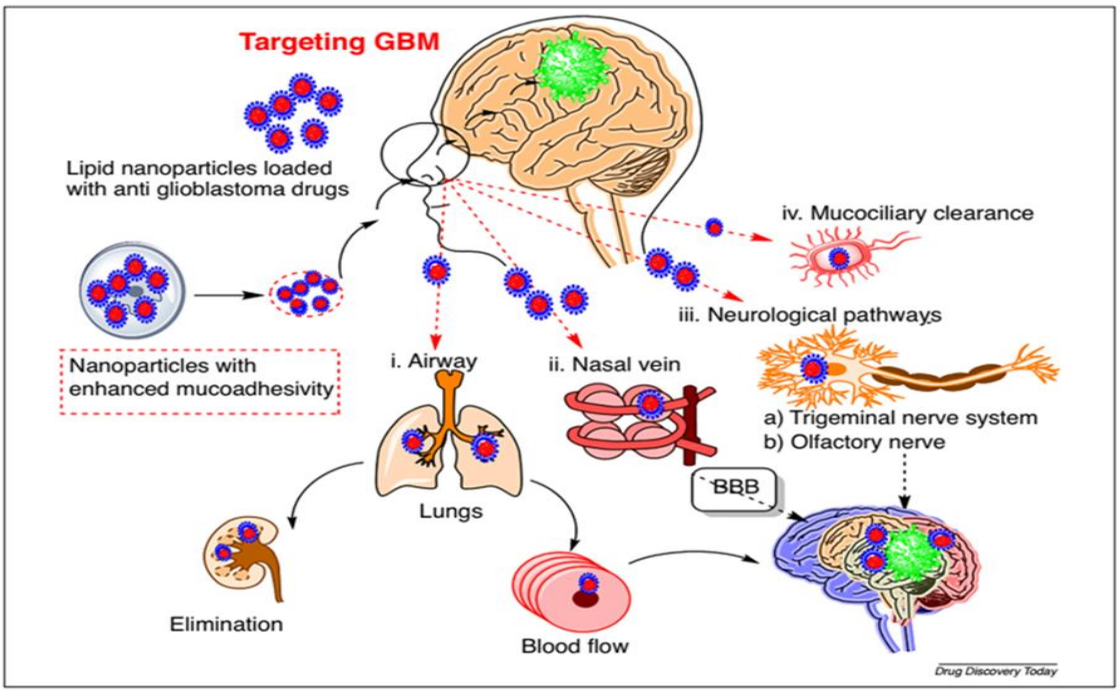

Figure 1 Possible mechanisms of lipid nanoparticles across the nasal membrane. The figure demonstrates that lipid nanoparticles with enhanced mucoadhesivity will follow four possible pathways: (i) Airway; (ii) Nasal vein; (iii) Neurological pathway; and (iv) Muco-ciliary clearance

2. AIMS OF THE WORK

This Ph.D. work aimed to prepare novel, innovative lipid nano-formulations containing anticancer drugs, which can be in the treatment of GBM through intranasal administration. The research work was constructed according to the following steps:

• Application of the QbD for the development of the lipid-nanoparticles with desired features for optimum targeting via intranasal administration.

• Screening and selection of PG and LOM to develop a suitable nasal product.

• To evaluate the in vitro physiochemical characterization of lipid nanoparticles.

• The characterization studies of final product PG-HA-coated liposomes, PG-SLNs, PG-Lipo, LOM-Lipo and PG-LOM Lipo.

• To analyze the PG anti-oxidant activity via scavenging assay.

• Comparative studies of the developed formulation (PG-Lipo, LOM-Lipo, PG-LOM Lipo) under simulated nasal conditions (in vitro cell line, ex vivo permeation and cell uptake study).

3. MATERIALS AND METFODS

3.1. Materials

3.1.1. Active pharmaceutical ingredients (APIs)

Lomustine (LOM) was purchased from Sigma-Aldrich (Budapest, Hungary). LOM has high lipophilic character (log P value of 2.16), molecular weight of 233.6 g/mol, pKa (13.3). Propyl gallate (PG) has low molecular weight (212.2 g/mol), greater lipophilic character (logP = 1.8), and pKa (7.94).

3.1.2. Excipients

Different types of excipients were used for the preparation of lipid nanoparticles loaded with anti-glioblastoma drugs. Cholesterol (biocompatible, inert, non-toxic), L-α-phosphatidylcholine (PC) from soybean low-density lipoprotein (asymmetric molecule with glycerol backbone and occurs naturally in lecithin of egg yolk), Tween 80 (polyoxyethylene sorbitan monooleate is a non-ionic surfactant.), and polyheptapeptides), Trehalose dihydrate (TRE) (Cryo-protectant).

3.2. Development and optimization of PG and LOM encapsulated lipid nanoparticles

3.2.1. *Screening of influencing factors of lipid nanoparticles loaded with PG*

QbD approach was used to screen the most critical factors that can affect the quality of final product. The QTPP (quality target product profile), CQAs (critical quality attributes), CPPs (critical process parameters) were determined.

3.2.2. *Risk Assessment (RA) strategy for PG-loaded lipid nanoparticles*

After determining the QTPP and selecting the critical factors (CQAs, CPPs), the next step is to perform the RA. The RA is the evaluation of interdependence between the QTPP elements and CQAs and between the CPPs and CQAs.

3.2.3. *Box-Behnken Design (BBD) on PG-Lipo*

Among the various methods of optimization, the BBD is the most broadly accepted and extrapolated in the design of the experimental phase of pharmaceutical formulation development.

3.2.4. *Response surface quadratic model Stat-Ease on PG-SLNs*

Stat-Ease Design Expert® version 10 (stat-Ease, INC.2021 East Hennepin Ave., Suite 480 software) was used to optimize the formulation process and product quality of PG encapsulated SLNs.

3.3. Development of Lipid nanoparticles

3.3.1. *Development of HA-coated liposomes*

A unique direct pouring method has been used to develop liposomes. This is a straightforward bottom-up size reduction process that produces a stable formulation with an optimal lipid and drug concentration. PG (12, 20, and 40 mg), PC (12, 24, and 32 mg), and cholesterol (8, 16, and 24 mg) were dissolved in 4 mL of an ethanol: acetone (3:1) combination and then added directly to the 10 mL aqueous phase. Using a hot-plate magnetic stirrer, the organic phase was evaporated at 60°C with constant stirring (400 rpm). The HA coating solution (50 mg/mL) was performed by soaking HA on the surface of purified water and waiting for 30 min with steady stirring at 400 rpm until it swelled completely. After that, 1 mL of HA solution was added drop-wise to the liposomal solution with a syringe for 30 min at room temperature with steady stirring at 700 rpm. After that, the mixture was kept in refrigerator at 8°C for 24 h. The liposomal formulation was centrifuged again at 4°C for 10 min at 25,000 rpm to remove the excess of HA which did not participate in electrostatic coating of liposomes, and the pellet was re-dispersed in 10 mL purified water.

3.3.2. *Development of PG-SLNs by modified injection method*

PG-SLNs were prepared through the modified injection method, where 10 mL of 0.2% w/v Tween 80 aqueous solution and 5 mL of the organic phase (ethanol:acetone 4:1) were added in different ratios. The PG (10 mg) and cholesterol (60 mg) were dissolved in a mixture of the organic phase and injected dropwise into surfactant solution under constant stirring at 700 rpm around 70 °C temperature.

3.3.3. *Preparation of co-encapsulated (PG-Lom) liposomes*

Preparation of co-encapsulated PG-LOM-Lipo was performed using direct pouring method. Different quantity of PG (12, 20, and 40 mg), LOM (12, 20, 40 mg), and cholesterol (8, 16, and 24 mg) were dissolved in 4 mL of an ethanol: acetone (3:1) mixture and directly added to 10 mL aqueous phase. The organic phase was evaporated at 60°C under constant stirring (400 rpm) using a hot plate magnetic stirrer.

3.4. Characterization of lipid nanoparticles

3.4.1. *Average hydrodynamic diameter, surface charge, and polydipersity index of lipid nanoparticles*

The Malvern nano ZS instrument was used to measure the average hydrodynamic diameter (Z-average), surface charge (zeta potential), and polydispersity index (PDI) of liposomes in folded capillary cells (Malvern Instruments, Worcestershire, UK).

3.4.2. *Gas chromatography-Mass Spectrometry for residual solvent determination (GC-MS) in liposomes*

For determination of acetone, Shimadzu GCMS-QP2010 SE (Shimadzu Europa GmbH, Duisburg, Germany) gas chromatography equipment with a 30 m long, 0.25-mm-diameter ZBWax-Plus column using He as the carrier gas was applied.

3.4.3. *Drug loading, Encapsulation efficiency, and Percentage Yield of lipid nanoparticles*

The centrifuge method was utilized to determine the encapsulation efficiency, % yield, and drug loading of PG-loaded lipid nanoparticles. Nanoparticles were initially centrifuged for 1 hour at 22,413 relative centrifuge force (RCF 16,500 rpm, 4°C). The supernatant was collected and screened using an Agilent 1260 HPLC (Agent Technologies, Santa Clara, CA, USA). The encapsulation efficiency, percent yield, and drug loading of PG and LOM loaded liposomes were all determined using the centrifuge method. The nanoparticles were initially centrifuged for 1 hour

at 22,413 relative centrifugal force in a Hermle Z323 laboratory centrifuge (RCF 16,500 rpm, 4°C). ChemStationB.04.03 software (Agilent Technologies, Santa Clara, CA, USA) was used to evaluate the data. PG has a 4 min retention time. The calibration line's linear regression was 0.998. In the instance of PG, the limit of detection (LOD) and the limit of quantification (LOQ) were 21 ppm and 63 ppm, respectively. While the quantification limit (LOQ) and detection (LOD) of LOM were 40 ppm and 13 ppm.

3.4.4. *Fourier Transform Infrared Spectroscopic (FTIR) measurement of lipid nanoparticles*

A thermo-Nicolet AVATAR FTIR spectroscopy (Thermo-Fischer, Waltham, USA) was used to gather FTIR spectra of pure drug, cholesterol, phospholipids, and coating material in the spectral region of 4000 and 400 cm^{-1}. The spectral resolution was adjusted to 2 cm^{-1}, and 128 scans were performed in case of all samples to improve the signal-to-noise ratio. A small amount of investigated sample (m<0.01 g) was mixed with 0.15 g of KBr, thereafter homogenized and milled. The powder mixture was pressed under pressing force of 10 kN using a Specac® hydraulic press (Specac Inc. Orpington, UK), with the pressing diameter of 13 mm. The background was also measured with pure KBr and subtracted form sample spectra.

3.4.5. *Differential Scanning Calorimetric (DSC) investigation of lipid nanoparticles*

To investigate the physiochemical properties and conversions, to analyze the crystallinity of solid-state products, DSC measurements were carried out. In this evaluation, drug loaded liposomes and SLNs were investigated to assess the possible intermolecular interactions between the drug, the HA-coated material, and lipids. DSC measurements were performed using a Mettler Toledo DSC 821e instrument (Mettler-Toledo GmbH, Greifensee, Switzerland). Approximately 3–5 mg of samples of physical mixtures and of components used in formulation development along with the coated material and product samples were loaded into an aluminum pan and examined in the scanning temperature range of 25–300 ℃, with an empty Al pan used as reference. The heating rate was 20 ℃/min in the presence of argon as a carrier gas with a flow rate of 150 mL/min. Each measurement was normalized to the sample size.

3.4.6. *X-ray Powder Diffraction (XRPD) investigation of lipid Nanoparticles*

The XRPD method was utilized for the structural characterization of pure drug and physical mixture compared to lipid nanoparticles. A BRUKER D8 Advance X-ray powder diffractometer (Bruker AXS GmbH, Karlsruhe, Germany) was used to obtain diffractograms. All of the results were obtained using a Cu Kλ1 slit-detector source (=1.5406). All of the formulation components, as well as the formulation itself, were scanned at 40 kV and 40mA in the angular range of 3-40°

2θ with a step time of 0.1 s and a step of 0.007 at a step time of 0.1 s. The samples were measured at the ambient temperature and humidity while examined in quartz holder.

3.4.7. *Surface morphology of lipid nanoparticles*

Transmission electron microscopy (TEM) (FEI Tecani G2 20 X Twin; FEI Corporate Headquarters, Hillsboro, OR, USA) was used to examine the surface properties of the liposomes formulations. Whereas the morphology and surface properties of PG-SLNs and the PG-SLNs were investigated using scanning electron microscopy (SEM) (Hitachi S4700, Hitachi Scientific Ltd., Tokyo, Japan).

3.5. *In vitro* characterization of lipid nanoparticles

3.5.1. *In vitro release study of lipid nanoparticles*

In vitro release study was performed under nasal conditions at 35°C using dialysis method under 50 rpm constant stirring. 1 mL of liposomal formulations were filled in 8 kDa dialysis bag (Spectra/Por®, Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) and placed in 50 mL of simulated nasal electrolyte solution (SNES) (which combined 2.98 g of KCl, 0.59 g of $CaCl_2$ anhydrous in 1000 mL of deionized water at pH 5.60). Aliquots were taken at predetermined time intervals. After filtration of withdrawn samples, both LOM and PG contents were determined via HPLC method as described above. All of the measurements were performed in triplicate manner. Data was presented as mean±SD. For each sample the *in vitro* release kinetics were also assessed using a variety of mathematical models, including Zero order, First order, Higuchi, Korsmeyer-Peppas, and Hixon-Crowel.

3.5.2. *In vitro permeability study of lipid nanoparticles*

For *in vitro* permeation studies, a modified horizontal side-bi-side® type diffusion apparatus was employed at 35°C using a circulating thermostat (Thermo Haake C10-p5, Sigma-Aldrich Co. LLC, St. Louis, MO, USA) under 100 rpm constant stirring of both chambers. An isopropyl myristate impregnated artificial membrane (0.45 um pore size, Pall Metricel cellulose membrane) with a 0.69 cm^2 diffusion surface was used to separate the donor and receptor compartments. The donor compartment contained 9 mL of pH 5.6 SNES, whereas the acceptor compartment contained pH 7.4 PBS. After every 5 min, aliquots were withdrawn from the acceptor phase and replaced with the same volume of fresh medium. Aliquots were assayed using HPLC to determine the amount of active compound diffused to the acceptor phase. Each formulation was measured in triplicate. Data was presented as mean±SD.

3.5.3. *In vitro Scavenging assay*

In 0.05 M phosphate buffer of pH 7.4 was prepared with (40 mM) hydrogen peroxide. In a hydrogen peroxide solution, PG liposomes with various drug concentrations (125, 250 and 500 ug/mL) were introduced (0.6mL, 40mM). After 10 minutes, the absorbance of hydrogen peroxide at 230 nm was measured against a blank solution of phosphate buffer without hydrogen peroxide.

3.6. Comparative study of the stable optimized formulations MTT Assay, *ex vivo* and Raman chemical mapping

3.6.1. *In vitro release of compared formulations*

In vitro release study was performed under nasal conditions at 35°C using dialysis method under 50 rpm constant stirring. 1 mL of liposomal formulations were filled in 8 kDa dialysis bag (Spectra/Por®, Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) and placed in 50 mL of simulated nasal electrolyte solution (SNES NaCl 7.5 mg/mL, 1.3 mg/mL of KCl, and 0.3 mg/mL of $CaCl_2$.2H2O). Samples were withdrawn in predetermined time intervals from release medium. After filtration of withdrawn samples, both LOM and PG contents were determined via HPLC method as described above.

3.6.2. *Ex vivo nasal diffusion study on rabbit nasal mucosa*

The ex vivo diffusion study of the formulation at nasal conditions was performed in a modified Side-bi-Side® horizontal diffusion cell. The following formulations were tested: LOM-Lipo, PG-Lipo, LOM and PG mixture solution, LOM solution and PG solution. Between the donor and the acceptor cell, rabbit nasal mucosa was placed. Both donor and acceptor cell volume were 9.0 ml for which the diffusion was investigated at 35 °C. The donor phase consisted of SNES and the acceptor phase was of pH 7.4 PBS. Sampling from the acceptor phase was performed at assigned time points (1, 3, 5, 10, 15, 30 and 60 min) and the drug concentration was measured via HPLC. The flux (J) was calculated from the quantity of the drug permeated through the membrane, divided by the surface membrane insert and the duration of experiment ($\mu g/cm^2/h$).

3.6.3. *Anti-proliferative MTT assay*

Anti-proliferative effect of LOM, PG and liposome complexes were determined under in vitro study using U87 glioblastoma cells, NIH-3T3 mouse embryonic and A2780 human ovarian cancer cells by means of MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) assay. Glioblastoma cells were cultivated in Dulbecco's Modified eagle's Medium supplemented with 1 mM sodium-pyruvate. NIH-3T3 and A2780 cells were cultured in Modified Eagle's Medium. Both

culture media were supplemented with 10% fetal bovine serum, 1% non-essential amino acids and an antibiotic-antimycotic mixture. A limited number of human cancer cells (5000/well) were seeded onto a 96-well micro-plate and became attached to the bottom of the well overnight. On the second day of the procedure, the test substances were added in serial dilution (applied in six different concentrations). After an incubation period of 72 h, the living cells were assayed by the addition of 20 μL of 5 mg/mL MTT solution. After a 4 h incubation, the medium was removed and the precipitated formazan was dissolved in 100 μL/well of DMSO during a 60-min period of shaking. Finally, the reduced MTT was assayed at 545 nm, using a micro-plate reader. Untreated cells were taken as the negative. All in vitro experiments were carried out on two 96-well dishes with at least five parallel wells in two independent experiments.

3.7. Statistical Analysis

The outcomes of this research data were statistically analyzed using Microsoft® Excel (Microsoft Office Professional Plus 2013) and JMP® 13 software (SAS Institute, Cary, CA, USA). One-way analysis of variance was used to analyze in vitro release and permeability data (ANOVA). A t-test was used for the in vitro scavenging assay. When the $P < 0.05$, difference were considered significant.

4. RESULTS AND DISCUSSION

4.1. Optimization of lipid nanoparticles

4.1.2. Initial knowledge space development of PG liposomes

There are various essential aspects impacting the final quality of the coated liposomal formulation as represented in the Ishikawa diagram (Figure 1).

4.1.3. *Results of Risk Assessment of PG-liposomes*

RA was used to follow the QbD-based formulation design and development after determining the impact (H, M, L) of the QTPPs and evaluating the CPPs and CQAs. For RA, a preliminary assessment of the dependency between QTPP elements and CQAs, as well as between CQAs and critical material attributes (CMAs/CPPs), was made using the previously mentioned 3-grade scale, as their impact on one another is high, medium, or low as shown in Figure 2.

4.1.4. *Design of Experiments: Box–Behnken Design*

The RA results aided in the design of the experiment. To screen the effects of the formulation parameters on the quality of the final coated liposomal formulation, the critical factors with the

greatest severity ratings were chosen. The quantity of phospholipids (L-phosphatidylcholine (PC)) and cholesterol (mg) selected from the CQAs, as well as the temperature (°C) of evaporation screened out from the CPPs, were the variables X_1 and X_2. The Z-average, PDI, and zeta potential were chosen as dependent factors according to our design strategy as shown in (Table 1).

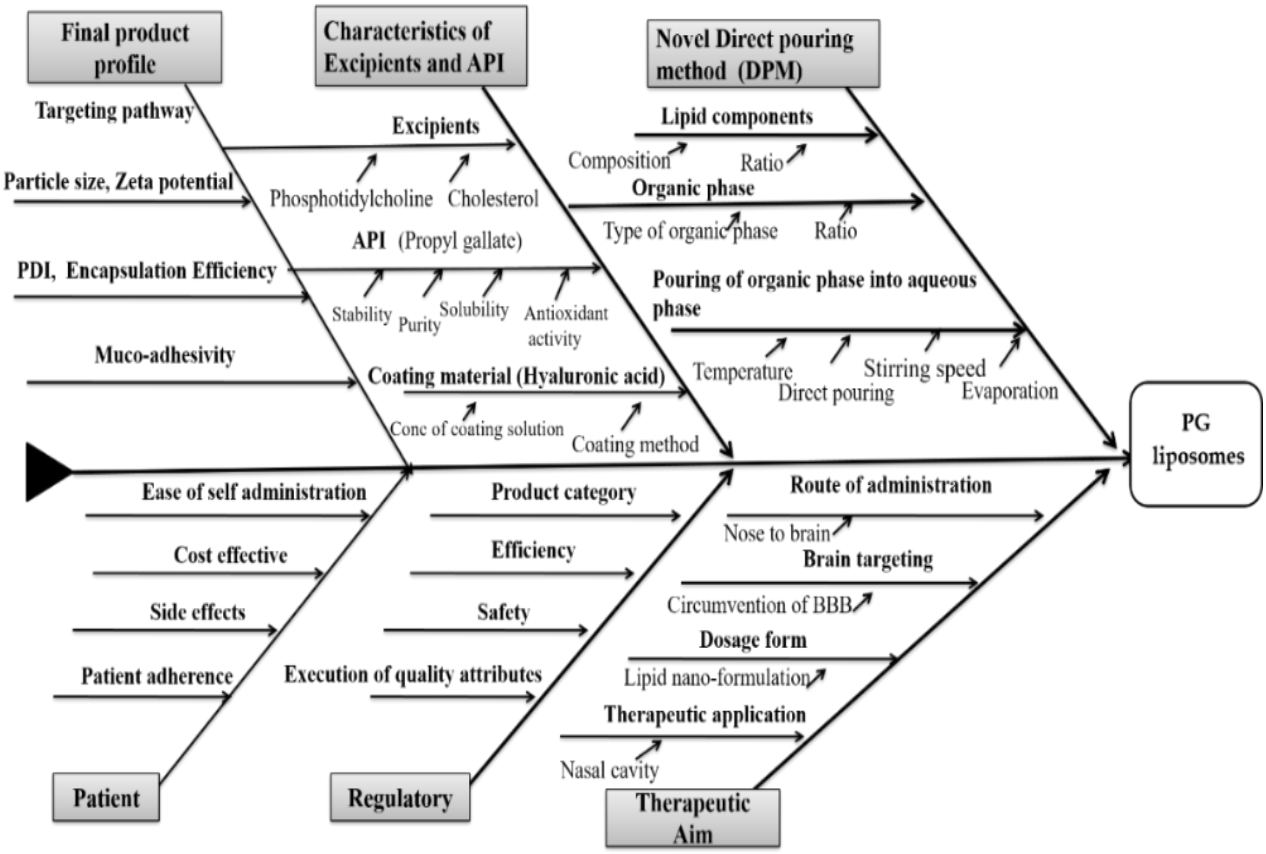

Figure 2. Ishikawa diagram showing the cause-and-effect relationships between influencing factors of hyaluronic acid (HA)-coated, n-propyl gallate (PG)-loaded liposomes for nose-to-brain drug delivery

Table 1. Z-average, polydispersity index (PDI), and zeta potential of 15 runs on the design of experiments

Number of Runs	Temperature (°C)	Amount of Phospholipids (mg)	Amount of Cholesterol (mg)	Z-Average (nm)	PDI	Zeta Potential (mV)*
1	50	16	16	150±10	0.27±0.01	-22±8.4
2	70	16	16	155±5.5	0.28±0.02	-18±6.5
3	50	32	16	145±4.5	0.29±0.02	-23±8.4
4	70	32	16	140±5.5	0.28±0.05	-24±8.4
5	50	24	8	125±6.6	0.25±0.07	-27±7.5
6	70	24	8	125±7.8	0.22±0.01	-28±8.5
7	50	24	24	400±22	0.40±0.08	-8±10.2

8	70	24	24	450±23	0.45±0.08	-7±12
9	60	16	8	121±24	0.24±0.09	-33±5.5
10	70	32	8	123±40	0.25±0.08	-28±6.5
11	60	16	24	430±20	0.55±0.05	-6±10
12	60	32	24	420±12	0.49±0.01	-8±10.2
13	60	24	16	130±10	0.21±0.02	-29±3.3
14	80	24	16	135±10	0.22±0.02	-26±5.5
15	70	24	16	142±8	0.27±0.01	-25±6.2

4.1.5. *Influence of Investigated Parameters on the Z-Average, PDI, and Zeta Potential*

The interdependence between the studied independent and dependent responses were explained by the significance of effects for each variable. The Z-average is closely related to particle size in colloidal drug delivery systems, therefore changes in it can signal changes in the primary particle size as well.

4.1.6. *Quality by Design Approach and Risk Assessment (RA)*

The particle properties of the nano-formulation were thoroughly evaluated during the RA process, as these are critical aspects. Quantification of these findings were done based on the interdependence rating. The interdependence rating assigned mostly high-grade scores when it came to particle characteristics (Z-average, PDI, and zeta potential), which is supported by the higher severity scores in Figure 3 when compared to the applicability affecting risk factors like muco-adhesivity, viscosity, and swelling properties.

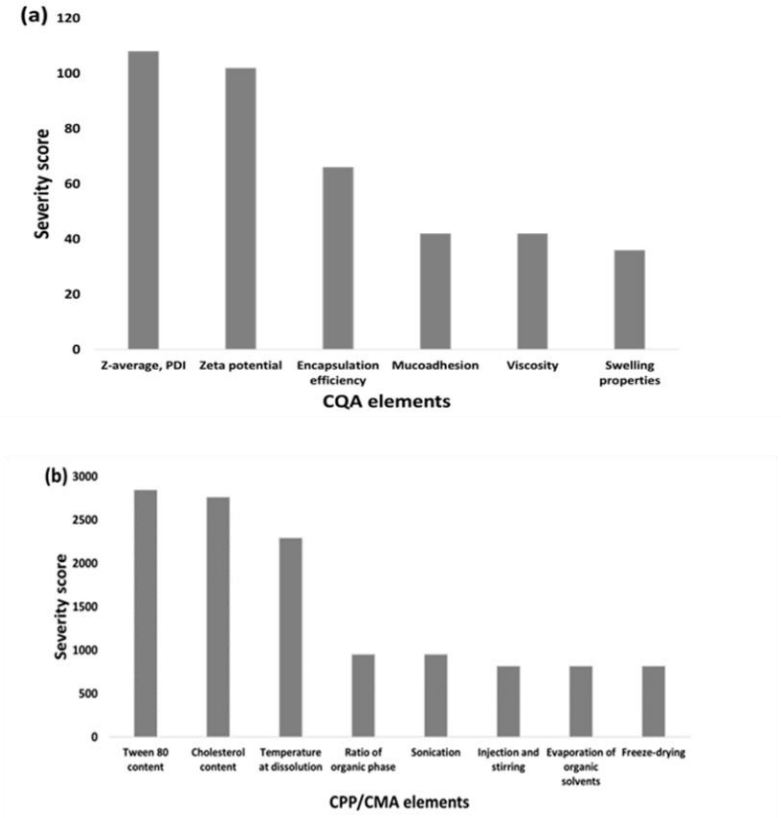

Figure 3. Probability rating of CQA (**a**) and CPP/CMA (**b**) elements. The Pareto charts are presented as the calculated severity scores assigned to the elements

4.1.7. *Central Composite Design (CCD)*

In a 15-formulations experiment series reported in, the effect of the factors with the greatest severity score, notably cholesterol content (A), Tween 80 content (B), and temperature (°C), on the independent factors: Z-average, PDI, and zeta potential, was studied. We entered the data into the software, and based on the smallest size, PDI, and the characteristic with the most negative zeta potential, a design expert chose run (7). Based on the lowest Z-average, PDI, and greater negative zeta potential, the software screened the optimal trial with a desirability of 0.99. Tween 80 and cholesterol in a 1:6 ratio were used in the improved PG-SLNs.

4.2. Characterization of Lipid nanoparticles

4.2.1. *Analysis of Z-Average, PDI, and Zeta Potential*

The Z-average, PDI, and zeta potential were measured both for optimized PG-loaded uncoated and HA-coated liposomes. (Table 2) illustrated the analysis of Z-average (nm), PDI and ZP (mV) of coated and uncoated nanoparticles. All the results met the standard set criteria, as mentioned above. The Z-average range was between 123 ± 2.5 nm and 300 ± 3.3 nm. The particle size of uncoated

liposomes was lower, as mentioned in the previous section, than that of coated ones, which showed that the HA coating of liposomes was successful.

Table 2. Analysis of particle size, PDI, and zeta potential of uncoated and coated liposomes

Formulations	**Z-Average *(nm)**	**PDI***	**Zeta Potential *(mV)**
Uncoated PG- liposomes	135.2±5.2	0.094±0.001	-29.9±5.8
Coated PG-liposomes	167.9±3.5	0.129±0.002	-33.6±4.5
PG-SLNs	103±46	0.16 ±0.001	-36 ±4.78

4.2.2. *Determination of acetone residual in the formulations fabricated via DPM*

As acetone is a Class 3 solvent, its residual content in the daily dose of the final product should be less than 5000 ppm, according to the International Conference on Harmonization (ICH) Q3C (R5) guideline for residual solvents. Gas chromatography was used to determine the residual acetone content in both coated and uncoated formulations shown in Table 3.

Table 3. Concentration of residual organic solvent in the optimized formulations.

Formulations	**Acetone**	**Maximum Residual Level* (ppm)**
Uncoated PG-liposomes	442 ppm	5000
Coated PG-liposomes	47 ppm	

*Based on the International Conference for Harmonisation (ICH) Q3C (R5)

Both results were acceptable under the established criteria of maximum allowable residual level, indicating that the DPM can be used to prepare liposomes.

4.2.3. *Encapsulation Efficiency, Percentage Yield, and Drug-Loading*

The encapsulation efficiency of PG-loaded liposomes was determined to be 90.6±0.47%. The percentage yield was 71.3±1.52%, indicating that the results of PG-loaded liposomes were in accordance with the desired parameters. The liposomes' drug-loading capacity was 2.81 %, which could be due to a higher amount of lipid used as the wall-forming agent.

4.2.4. *Fourier-Transform Infrared Spectroscopy (FTIR) investigations*

The FTIR spectra of the components of coated and uncoated liposomes are presented in Figure 4a. Compatibility study results showed that there was no interaction between the formulation components.

4.2.3. *Differential Scanning Calorimetric (DSC) Analysis Results*

The thermal behavior of PG, cholesterol, PC, HA, and liposomes coated and uncoated with HA was investigated with DSC (Figure 4b). The endothermic peaks at 150°C on the DSC curves of PG and cholesterol correspond to their melting points. PC and HA did not exhibit any distinct melting event, possibly due to their non-crystalline nature.

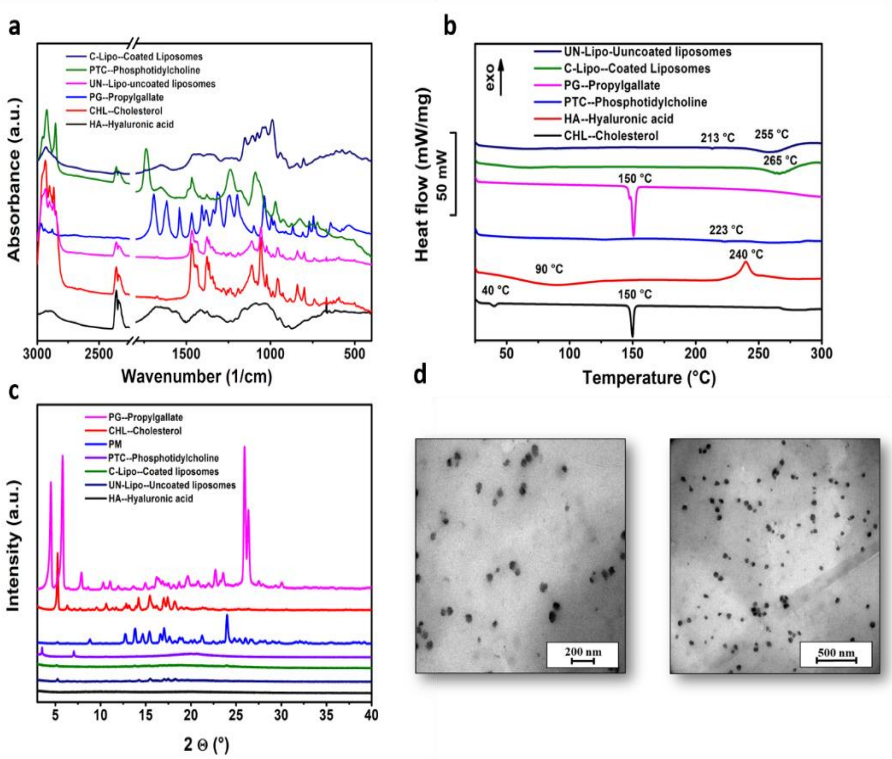

Figure 4. Fourier-transform infrared spectroscopy (FTIR) spectra (**a**), differential scanning calorimetry (DSC) thermo-grams (**b**), and X-ray powder diffraction (XRPD) diffractograms of coated and uncoated liposomes and components of liposomal formulation (**c**), and transmission electron microscopy (TEM) images of liposomes at different resolution scales (**d**).

4.2.4. *X-ray Powder Diffraction (XRPD) analysis results of PG-Lipo and PG-SLNs*

XRPD studies showed the crystalline nature of PG and cholesterol and the amorphous nature of PC and HA as coating materials in accordance with the DSC results, as shown in Figure 4c and 5a.

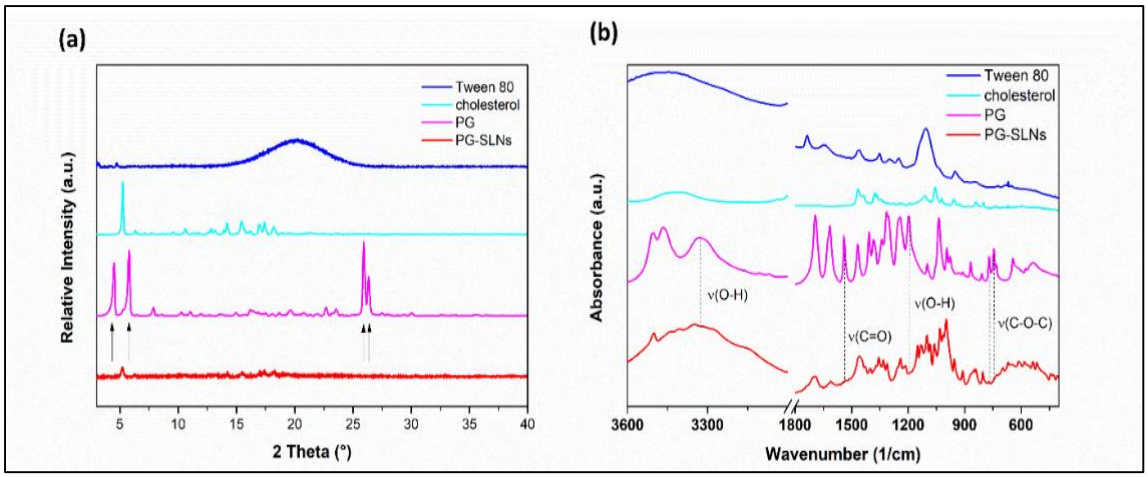

Figure 5. XRPD diffractogram (**a**) and FTIR spectra (**b**) of PG-SLNs and their components

In the diffractograms of uncoated and coated liposomes, the characteristic peaks of cholesterol (at 5.18, 13.8, 15.34, 16.6, 17, and 17.6 2θ) as a wall-forming agent can be observed, but in the case of coated liposomes, these peaks disappeared.

4.2.5. *Morphology of liposomes and PG-SLNs*

The transmission electron microscopy (TEM) images of PG-loaded liposomes with coating and without coating showed the spherical shape and uniform distribution of liposomes, as shown in Figure 4d, showing a mono-disperse distribution without any aggregation.

SEM images of lyophilized PG-SLNs, shown in Figure 6, also proves that the nanoparticles have spherical morphology and are homogenously distributed in the gel structure.

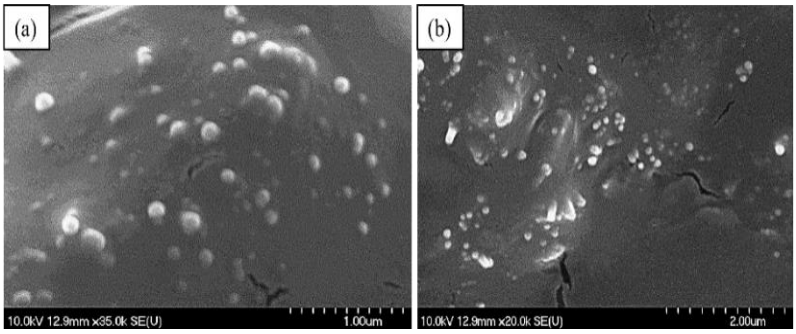

Figure 6. SEM images of optimized lyophilized PG-SLNs (**a,b**)

4.3. Results of in vitro characterizations of PG-Lipo and PG-SLNs

4.3.1. *In Vitro release studies of PG-Lipo*

In vitro release studies of uncoated and HA-coated liposomes showed 75% and 60% drug release within 24 h, respectively, in simulated nasal electrolyte solution (SNES) (pH 5.6), as shown in Figure 8a, while 80% of the drug was released within 48 h from coated liposomes. The release of PG from solution was significantly lower (10% within 24 h) in comparison to other liposomal

formulations. Drug release kinetics was determined by fitting kinetic models, and data were evaluated by the correlation coefficient (R^2) (Table 4).

Table 4. R^2 values of drug release from uncoated and HA-coated liposomes

Kinetic Model	Zero Order	First Order	Korsmeyer-Peppas	Higuchi	Hixon-Crowel
R^2 Value of uncoated liposomes	0.7211	0.8922	0.8656	0.9238	0.8691
R^2 value of coated liposomes	0.745	0.8935	0.8662	0.9073	0.8448

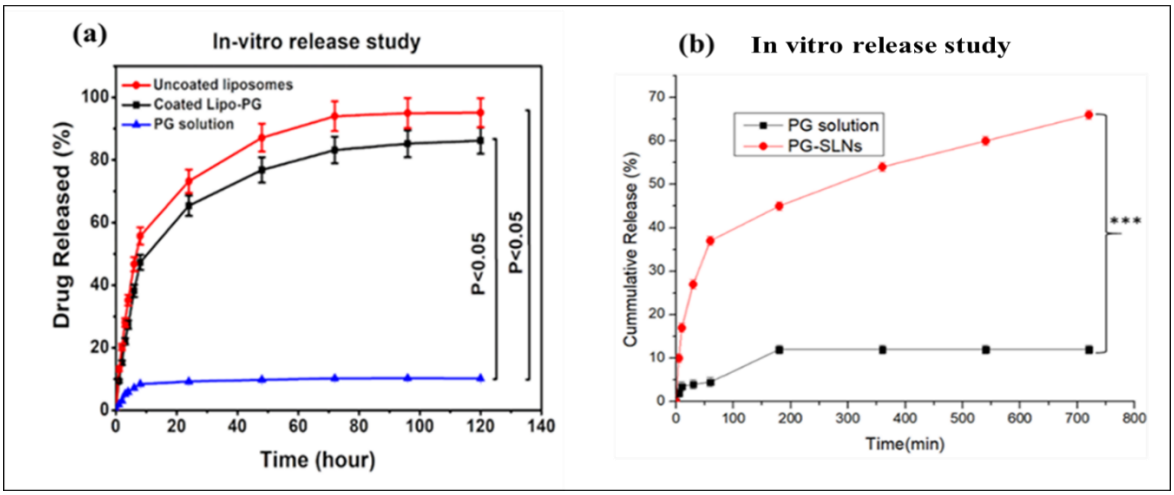

Figure 7. *In vitro* release study of PG in phosphate buffer (pH 5.6) (**a**) and in vitro permeability studies of uncoated liposomes, coated liposomes, and PG solution (**b**)

4.3.2. *In Vitro permeability study of PG-Lipo*

The modified side-bi-side apparatus was used for the in vitro nasal permeation study of uncoated and coated PG-loaded liposomes and the PG solution. Figure 8b shows the rate of PG permeation from the donor to the acceptor phase. The maximum permeation of PG from coated liposomes was 420 µg/cm^2 after 60 min. The PG-containing uncoated liposomes provided faster diffusion and higher drug concentration (>500 µg/cm^2 after 60 min) in comparison to the PG solution. In case of the PG solution, permeation was negligible, about 5 µg/cm^2. However, when compared with coated liposomes, there was a difference of 80 µg/cm^2 among the permeation values.

4.3.3. *In Vitro permeation of PG-SLNs*

The *in vitro* nasal permeation investigation used a modified Side-bi-Side® equipment to examine the diffusion of PG solution, PG-SLNs. Figure 8a illustrates the cumulative PG penetration from

the donor to acceptor phase through an isopropyl myristate-impregnated synthetic cellulose membrane. After 60 min, the cumulative permeation of PG from PG-SLNs was 190 μg/cm^2.

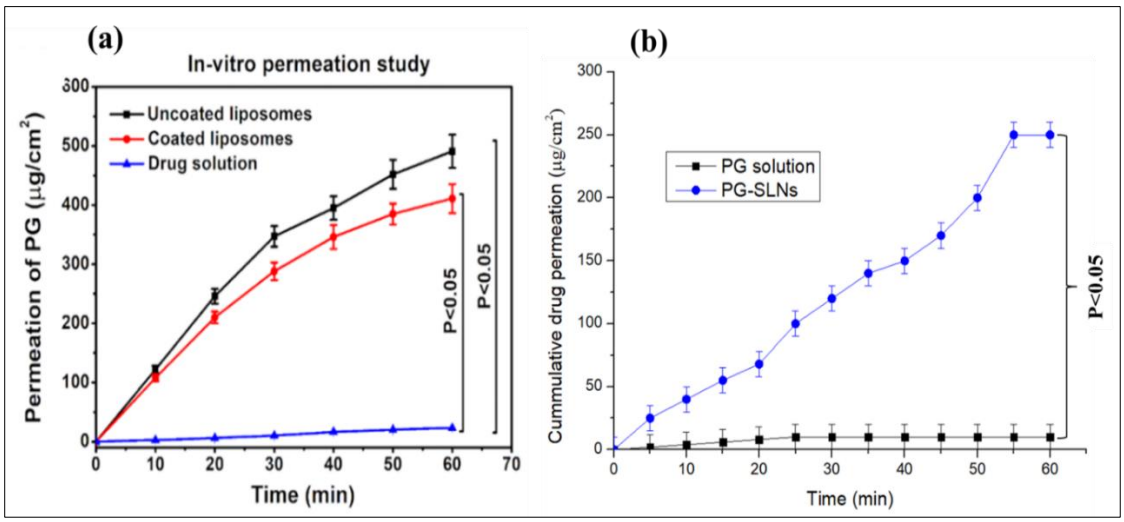

Figure 8. *In vitro* permeation of pure PG solution, uncoated liposomes, coated liposomes (**b**). PG solution, PG-SLNs Data are means ± SD (n = 3 independent measurements).

4.3.4. *Anti-oxidant activity measurement with hydrogen peroxide (H_2O_2)*

Coated and uncoated liposomes containing PG in different concentrations were screened for in vitro scavenging activity using hydrogen peroxide. The scavenging activity of the formulations and the initial PG solution (as a control) are presented in (Figure 9a).

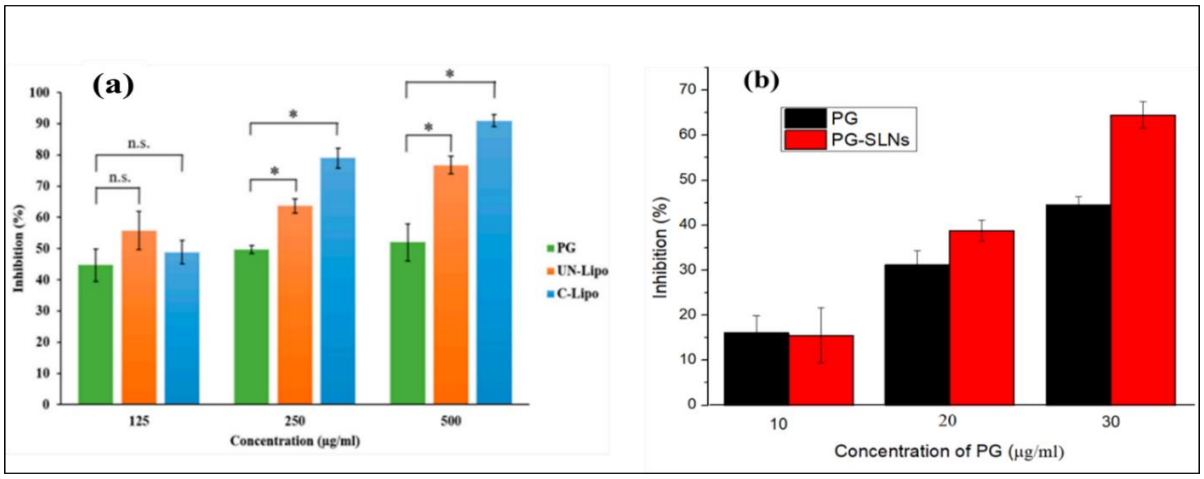

Figure 9. Percentage inhibition of hydrogen-peroxide-scavenging activity of different concentrations of PG-containing coated and uncoated liposomes (**a**). Percentage inhibition of the hydrogen-peroxide-scavenging activity of different concentrations of PG-containing SLNs (**b**).

It was revealed that the liposomal formulations preserved the antioxidant activity of PG based on the scavenging activity measurement against H_2O_2. With increase in concentration of PG the percent inhibition was enhanced. (Figure 10b).

4.4. Comparative in vitro and ex vivo studies of Optimized formulations

4.4.1. *In vitro release study of compared formulation (LOM-Lipo, PG-Lipo, LOM-PG Lipo)*

Drug release kinetic of liposomal formulations is a crucial part of the rational design, predicting the subsequent characteristics of drug delivery system after administration. The *in vitro* release profile reveals (Figure 10) important information on the structure and behavior of the formulation, possible interactions between the drug and lipid composition, and their influence on the rate and mechanism of drug release. The dialysis-based release method is a well-established and useful technique to study *in vitro* release from nano-particulate delivery systems.

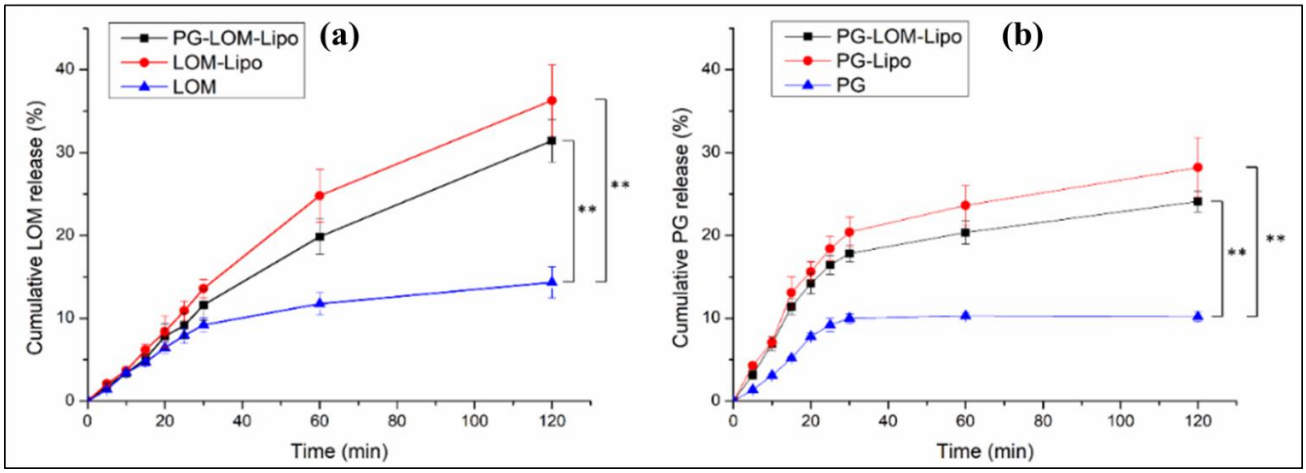

Figure 10. Cumulative drug release of LOM- (a) and PG-containing (b) formulations in comparison to initial LOM and PG suspension. ANOVA test was performed to check the significance of the differences between liposomal formulations and initial drug suspension.

4.4.2. *In vitro cell line studies (anti-proliferative assay results)*

IC_{50} values of the tested formulations and their 95% confidence intervals (CI) was calculated. LOM Lipo, PG Lipo, LOM-PG Lipo formulations were tested on murine fibroblast cells and human glioblastoma cell lines (U87) for the anti-proliferative effects with MTT assay. The cells were examined in 96-well plates in water-based culture media. The combination of PG-LOM Lipo showed highest anti-proliferative activity in comparison to other formulations. In contrast to other cell lines A2780 show more sensitivity among tested formulations.

Table 6. IC50 values of the tested formulations Lomustine, PG, PG-LOM (DMSO), LOM Lipo, PG Lipo, LOM-PG Lipo

Compounds	IC_{50} values (µM) [95% CI]		
	NIH	U87	A2780
LOM (DMSO)	264.80 [220.3-318.3]	35.09 [31.02-39.69]	41.88 [35.74-49.09]
PG (DMSO)	29.14 [25.10-33.83]	59.42 [51.67-68.32]	14.64 [12.97-16.53]
LOM (Liposome 10 mg/5 ml)	>500	>500	>100
LOM (Liposome 10 mg/10 ml)	>500	>500	>100
PG (Liposome)	62.93 [56.83]	275.3 [246.8-307.2]	49.00 [44.24-54.27]
LOM-PG-Liposome *	140.20 [125.30-156.80]	217.60 [190.60-248.40]	70.45 [63.55-78.10]

4.4.3. *Ex vivo Raman maping*

Passive trans-mucosal uptake of liposomal formulations was studied by Raman mapping. Isolated rabbit mucosa was treated with PG-Lipo, LOM-Lipo, PG-LOM-Lipo, PG solution and LOM solution, and the penetration depth was analysed and compared with non-treated nasal mucosa specimen using Raman correlation mapping (Fig. 11). The mucosal distribution correlation maps show a remarkable Raman intensity on the top of the nasal mucosa specimens in both cases (non-treated, treated), which can correspond to the high protein content of the epithelial layer.

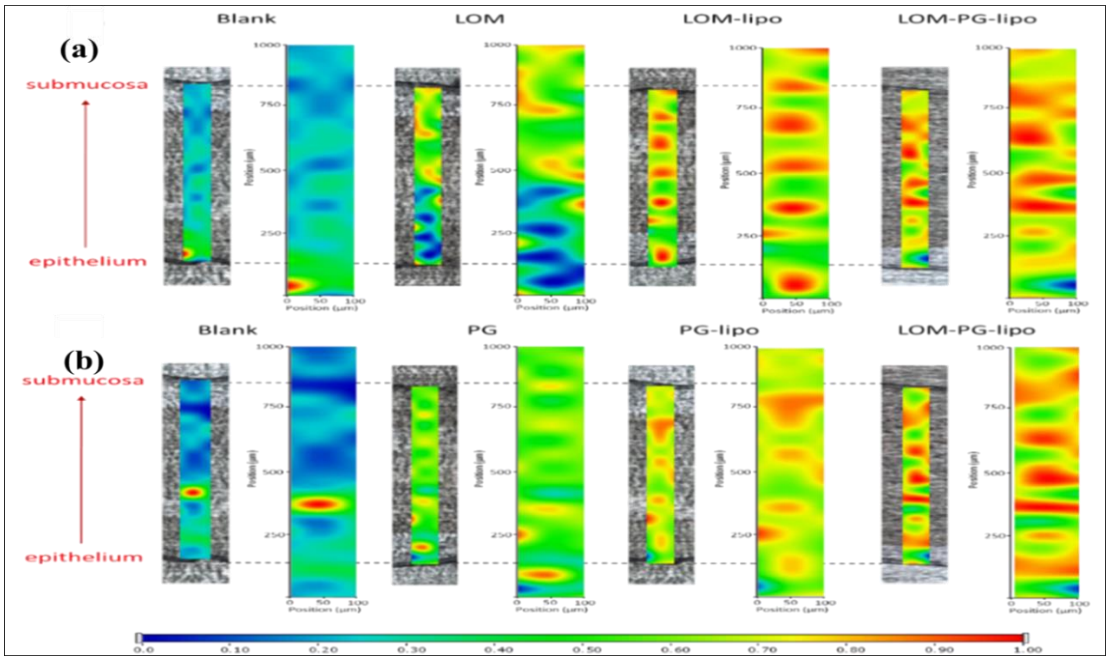

Figure 11. Raman correlation maps of the distribution of LOM (A) and PG (B) in the rabbit nasal mucosa compared to the non-treated nasal mucosa specimen.

4.4.4. Cellular Uptake of Propidium–Iodide Labelled Liposomes

As propidium-iodide (PI) is not membrane permeable, it is a suitable indicator for investigating cellular uptake. By loading PI as a chemical marker in the liposomal carrier, the endocytosis of liposomes can be visualized. Figure 12 shows the fluorescent microscopic results of U87 and U251 cells treated with PI-loaded liposomes (PI-Lipo) for 24 h.

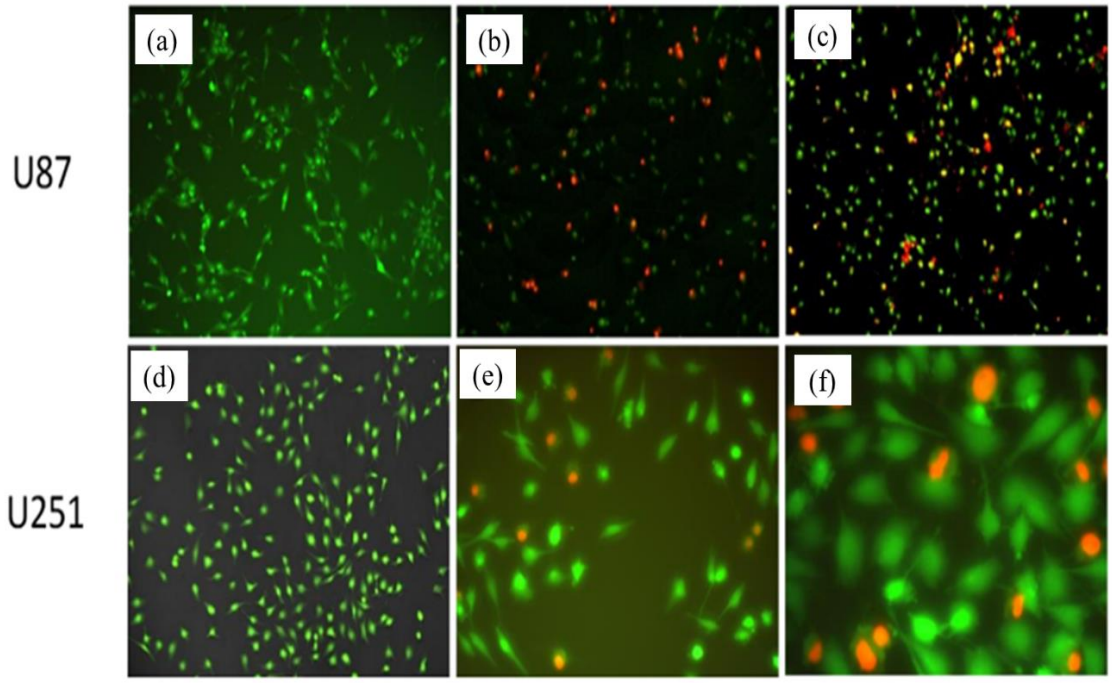

Figure 12 Fluorescence imaging of PI-Lipo cellular uptake on U87 (**b**,**c**) at 10× magnification and U251 cell line at 20× (**e**) and 40× magnification (**f**) after 24 h incubation compared to control group of U87 (**a**) and U251 (**d**) stained with cell track green

5. CONCLUSIONS

The DPM as a novel liposome preparation method has not been previously applied for the preparation of liposomes. As a gentle formulation method, it is suitable for loading anti-oxidants into liposomes, preserving their stability. The study helped in the preparation of coated liposomes, and PG was used as the model compound that has proven anti-cancer efficacy. PG-SLNs were successfully prepared using a modified injection method. Following the QbD approach for the optimization of formulation and process parameters affecting the quality of the nano-system, the initial risk assessment study and the design of experiment were applied. The co-encapsulation of PG and LOM endowed the liposomes with the ability to facilitate the synergistic anti-cancer activity with significant potential to be co-encapsulated with other anti-glioblastoma drugs. Our optimized platform provided in vitro proof of the potential of combining the advantages of PG and

LOM into liposomes as a promising intranasal delivery system. PG and LOM co-encapsulated liposomes were compared against PG-Lipo, LOM-Lipo. The optimized formulation of PG-LOM Lipo has Z-average of (172±2.5 nm), PDI (0.149±0.002), ZP (-36.6±4.5 mV).

6. NOVELTY OF WORK

The conventional techniques for liposome development and size reduction are convenient to use with sophisticated equipment. However, problems related to scale-up and scale-down applications have encouraged improvements to conventional processes that led to the development of novel methods for liposome development. Applying the novel DPM avoids the use of any additional size reduction or extrusion technique, which makes the preparation process more feasible and precise and less time consuming. There is no reported findings are present regarding the encapsulation of PG into nanoparticles and no data regarding the synergistic combination of PG-LOM is present in literature. The following studied (LOM and PG) combination may prove as an effective anti-cancer treatment for targeting glioblastoma multiforme.

7. Financial Support

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2. **Sabir, Fakhara,** Gábor Katona, Edina Pallagi, Dorina Gabriella Dobó, Hussein Akel, Dániel Berkesi, Zoltán Kónya, and Ildikó Csóka. 2021. 'Quality-by-Design-Based Development of n-Propyl-Gallate-Loaded Hyaluronic-Acid-Coated Liposomes for Intranasal Administration', Molecules (Basel, Switzerland), 26: 1429.
3. **Sabir, Fakhara,** Gábor Katona, Ruba Ismail, Bence Sipos, Rita Ambrus, and Ildikó Csóka. 2021. 'Development and Characterization of n-PropylGallate Encapsulated Solid Lipid Nanoparticles-Loaded Hydrogel for Intranasal Delivery', Pharmaceuticals, 14: 696.