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**Master's degree in INDUSTRIAL NANOBIOTECHNOLOGIES FOR
PHARMACEUTICALS**

Thesis title:

Development and Characterization of Lyophilized Drug-Loaded Liposomes:

From Electroporation-Enhanced Release to Storage Stability

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Abbreviations

DLS: Dynamic light scattering

EE%: encapsulation efficiency

EP: electroporation

GS: gentamicin-sulfate

NTA: Nanoparticle tracking analysis

PDI: polydispersity index

PLC: placebo

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1. Introduction

Liposomes are widely investigated nanocarriers for drug delivery due to their biocompatibility, biodegradability, structural versatility, and ability to encapsulate pharmaceutical compounds with different physicochemical properties [1]. They consist of one or more phospholipid bilayers surrounding an aqueous core, allowing hydrophilic compounds to be incorporated within the internal aqueous compartment, while hydrophobic and amphiphilic molecules can associate with the lipid bilayer [2]. Liposomal encapsulation can protect therapeutic agents from degradation, modify their pharmacokinetic behavior, and provide controlled drug release, potentially reducing exposure of non-target tissues to the free drug [2,3].

The physicochemical properties of liposomes can be tailored through parameters such as lipid composition, cholesterol content, surface charge, particle size, and lamellarity. In addition, the preparation method strongly influences the characteristics of the resulting formulation. Microfluidic technologies provide controlled and continuous mixing of lipid and aqueous phases, enabling reproducible liposome production with control over particle size and size distribution. Their scalability and reproducibility make microfluidic approaches particularly attractive for pharmaceutical formulation development [4].

Despite these advantages, maintaining liposome stability during prolonged storage remains a major challenge. In aqueous dispersions, liposomes may undergo physical instability, including aggregation and fusion, as well as chemical degradation through phospholipid hydrolysis and oxidation [5,6]. These processes can alter particle size and membrane permeability and promote leakage of encapsulated drugs, ultimately reducing drug retention and formulation reproducibility. Such leakage is particularly relevant for hydrophilic compounds located within the aqueous core, where loss of membrane integrity can directly affect the retained drug concentration [6]. Environmental factors, including temperature, pH, oxygen exposure, moisture, and freeze–thaw cycles, can further influence these degradation processes, highlighting the importance of appropriate storage conditions [7].

Lyophilization is widely employed to improve the storage stability of liposomal formulations by converting aqueous dispersions into dry products [8]. Removal of water can reduce degradation processes and facilitate storage, transportation, and handling. However, freezing and dehydration themselves expose liposomes to stresses associated with ice formation, osmotic changes, and membrane dehydration, potentially causing aggregation, fusion, membrane disruption, and drug leakage [6]. Cryoprotectants such as trehalose, sucrose, and mannitol are therefore commonly incorporated to protect the phospholipid bilayer during freeze-drying and subsequent reconstitution [6,9]. Following lyophilization, the ability to recover the physicochemical characteristics of the original formulation is an important indicator of successful preservation. Characterization after reconstitution commonly includes particle size and size distribution, particle concentration, encapsulation efficiency, and drug retention [9]. Monitoring these properties over time and under different storage conditions is therefore essential to establish the stability profile of a lyophilized liposomal formulation.

Beyond storage stability, effective drug delivery requires control over the release of the encapsulated therapeutic agent [10,11]. Stimulus-responsive liposomes provide an approach in which drug release can be modulated by an externally applied trigger. Among these strategies, electroporation uses short electric field pulses to induce transient increases in membrane permeability [12]. Because liposomal phospholipid bilayers share structural features with biological membranes, electrical stimulation can also influence liposomal membrane permeability and thereby promote the release of encapsulated compounds [12].

Combining liposomal delivery with electroporation is therefore of interest as a strategy for externally modulating drug release. Electroporation may facilitate both the release of therapeutic agents from liposomal carriers and their uptake by electrically permeabilized cells, providing a potential link between controlled release and enhanced local drug delivery [12,13]. However, the behavior of electroporated liposomes can also depend on the surrounding environment, including pH, ionic composition, and the presence of biological components. Evaluating drug release under progressively more physiologically relevant conditions is consequently important when assessing the performance of electrically responsive liposomal systems.

2. Aim

This study aimed to develop and characterize a lyophilized gentamicin sulfate (GS)-loaded liposomal formulation produced by microfluidics and to evaluate its drug-release performance and long-term stability. The effect of lyophilization was first assessed by comparing the physicochemical characteristics and encapsulation efficiency of the formulation before and after freeze-drying and reconstitution.

A preliminary *in vitro* release study was then performed to compare two formulations differing in their microfluidic production parameters and select the formulation exhibiting the most favorable release behavior. The selected formulation was subsequently investigated under different release media and pH conditions following electroporation to evaluate electrically modulated gentamicin release.

Finally, the long-term stability of the lyophilized formulations was assessed under different storage temperatures and timepoints by monitoring particle size, particle concentration, and gentamicin retention. Collectively, the study aimed to identify a formulation capable of maintaining its physicochemical properties during storage while retaining responsiveness to electrically triggered drug release to be explored for therapeutic purposes.

3. Materials and Methods

3.1. Materials

The following reagents and materials were used in this study: DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine, MW 790.15 g/mol), Cholesterol (MW 386.65 g/mol) and DOTAP (1,2-dioleoyl-3-trimethylammonium-propane, MW 698.54 g/mol) were purchased from Avanti Polar Lipids Inc. (Alabaster, United States). D-(+)-Trehalose dihydrate (MW 378.33 g/mol), Gentamicin sulfate powder, Ninhydrin (MW 178.14 g/mol), PBS tablets, and Triton X-100 were obtained from Sigma-Aldrich (Milan, Italy). Ethanol 96% v/v was supplied by AppliChem GmbH (Darmstadt, Germany). Spectra/Por dialysis membranes (MWCO 12–14 kDa) were from Spectrum Labs via Fisher Scientific (Waltham, USA). Stir bars (PTFE) were from Heathrow Scientific. Distilled and ultrapure Milli-Q water were provided by Merck Millipore (Burlington, MA, USA). For analysis, Malvern

Panalytical Folded Capillary Zeta Cells (10038296) and Sarstedt cuvettes (10 × 10 × 45 mm) were used. Sterile filters (Millex-GP 0.22 µm, PES membrane) were from Merck Millipore Ltd. *E. coli* ATCC 25922 was obtained from St. James Microbiology Department. Human serum Albumin (HSA) (BioReagent, ≥96% purity, A1653; Sigma-Aldrich, St. Louis, MO, USA).

3.2. Method

3.2.1. Design of experiment

Two GS-loaded liposomal formulations were included in the experimental design. Both formulations have the same lipid composition (DSPC: Chol: DOTAP), aqueous GS concentration, and trehalose content, as reported in [Table .1](#), but differed in the microfluidic flow-rate ratio (FRR) and total flow rate (TFR). FRR is the volumetric ratio of the aqueous phase to the organic lipid phase entering the micromixer, whereas TFR is the combined volumetric flow of both phases through the cartridge.

During the initial formulation-development phase conducted in Pavia, the 5:1 formulation was developed and subjected to physicochemical characterization and encapsulation-efficiency assessment before and after lyophilization. Subsequently, during the experimental work conducted at TTMI (at TCD, Ireland), the 5:1 formulation was compared with a previously developed 3:1 GS-loaded liposomal formulation available at TTMI and described by Costanza Di Marco (2025) [14]. A preliminary *in vitro* release study was performed under identical experimental conditions to compare the release performance of the two formulations and determine which formulation should be carried forward for the subsequent investigations. Based on the resulting release profiles, the 5:1 formulation was selected for the extended *in vitro* release and long-term stability studies.

Table 1. Composition of GS-loaded liposomes and microfluidic process parameters

	GS-liposome (3:1)	GS-liposome (5:1)
Lipid Composition ratio DSPC:Chol:DOTAP	67.5:27.5:5	67.5:27.5:5
Aqueous Composition Gentamicin Sulfate	1 mg/ml	1 mg/ml
FRR	3:1	5:1
TFR	12 ml/min	10 ml/min
Cryoprotectant	Trehalose 300 mg/ liposome batch	Trehalose 300 mg/ liposome batch

GS-loaded liposomes were prepared using a microfluidic mixing technique with a Nanoassembler® Benchtop instrument (Precision NanoSystems Inc., Vancouver, Canada) equipped with a staggered herringbone micromixer (SHM) cartridge. The lipid phase consisted of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol (Chol), and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) dissolved in absolute ethanol to obtain a total lipid concentration of 10 mM. The lipid molar ratio used throughout this study was 67.5:27.5:5 (DSPC: Chol: DOTAP).

Placebo and GS-loaded liposome batches were prepared; they differ in the aqueous phase. Step by step of the two batches preparation is presented in [Figure 1](#), liposomes encapsulated in gentamicin consisted of phosphate-buffered saline (PBS, pH 7.4) containing GS at a concentration of 1 mg/mL, while for placebo liposomes, PBS without gentamicin was used under identical preparation conditions.

Prior to liposome preparation, the lipid and aqueous solutions were loaded separately into syringes connected to the NanoAssemblr® system, as shown in [Figure 1](#). Liposomes were produced by controlled mixing of both phases inside the microfluidic cartridge using a flow rate ratio (FRR) of 5:1 (aqueous phase: organic phase) and a total flow rate (TFR) of 10 mL/min.

Immediately after microfluidic mixing, the liposomal suspension was collected and purified by centrifugation at 16,400 rpm for 30 minutes at 4°C to remove non-encapsulated gentamicin. Following centrifugation, the supernatant was collected and stored at 2°C and protected from light until analysis, while the liposomal pellet was resuspended in 1 ml PBS for proceeding with characterization. Dynamic Light Scattering (DLS) Nicomp 380 ZLS Particle Sizing System (Particle Sizing Systems, CA, USA) was initially used in University of Pavia, while further characterization was carried out at TTMI (Dublin, Ireland) using three complementary analytical instruments: Dynamic Light Scattering (DLS) (Malvern Instruments, Malvern Panalytical, Zetasizer, UK), Nanoparticle Tracking Analysis (NTA) NanoSight PRO (Malvern Panalytical, UK) and VideoDrop (Myriade, Paris, FR). The preparation step by step protocol for samples having a flow rate ratio (FRR) of 3:1 and total flow rate (TFR) of 12 ml/min is provided in the previous work carried out by “Costanza Di Marco in 2025 as part of her INBP master thesis”.

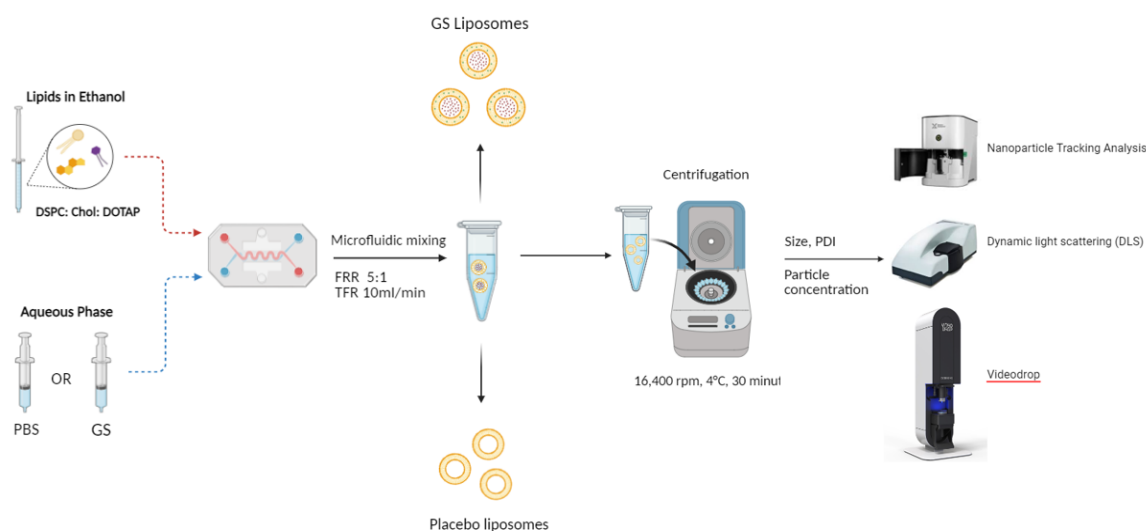


Figure 1. Placebo and GS- loaded liposomes preparation procedure using microfluidic technique along with the characterization of the batches with three different instruments Malvern DLS, Malvern-Panalytical NTA PRO and VideoDrop.

3.2.2. Lyophilization of the liposomes

Following centrifugation, the resuspended pellets were subjected to freeze-drying to improve their long-term stability and facilitate storage. Prior to freeze-drying, 300 mg of trehalose was added to each liposomal suspension as a cryoprotectant. After the addition of trehalose, the liposomal suspensions were gently vortex-mixed until complete dissolution of the cryoprotectant

was achieved. The samples were subsequently frozen at -25°C for 3 to 4 hours. Lyophilization was performed using a Lio® 5 Pascal freeze dryer (5Pascal S.r.l., Milan, Italy) under a chamber pressure of 0.3 mbar and a condenser temperature of -50°C for 24 hours ([Figure 2](#)). Upon completion of the freeze-drying cycle, a dry lyophilized cake was obtained and stored under the appropriate storage conditions until further analysis [[6,7](#)]. Ten batches were prepared to be used at the Trinity Translational Medicine Institute (Dublin, Ireland), following the process presented in [Figure 2](#).

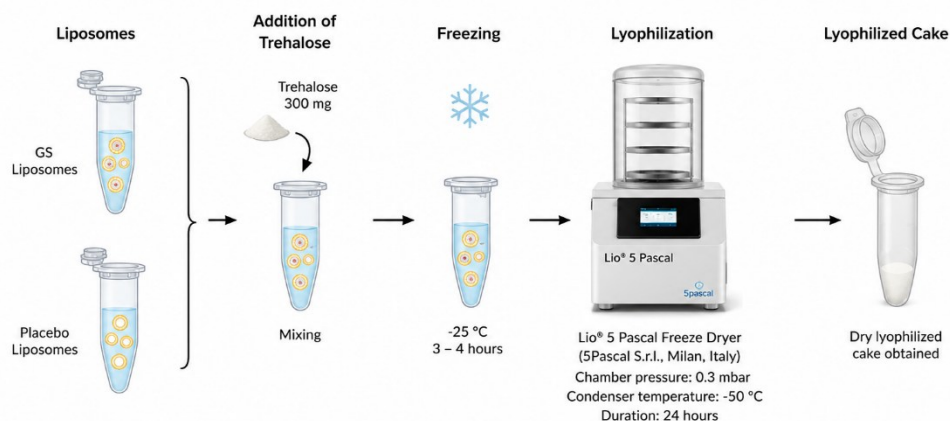


Figure 2. Freeze-drying procedure for both placebo and GS- liposomes to formulate dry lyophilized liposomes

3.2.3. Encapsulation Efficiency (EE%)

The encapsulation efficiency (EE%) of the 5:1 GS-loaded liposomal formulation was determined before and after lyophilization as part of the initial formulation characterization performed in Pavia, to evaluate the effect of the freeze-drying process on drug retention.

For the non-lyophilized formulation, the liposomal pellet obtained following the preparation procedure ([section 3.2.1](#)) was directly resuspended in 2% (v/v) Triton X-100 prepared in PBS to disrupt the phospholipid bilayer and completely release the encapsulated gentamicin. The suspension was vortex-mixed for 2 min at 2,400 rpm, followed by sonication for 15 min to ensure complete liposome lysis and drug extraction. For the lyophilized formulation, the freeze-dried liposomal powder was first reconstituted in 1 mL of PBS (pH 7.4) to restore the original formulation volume and subjected to magnetic stirring at 300 rpm for 30 min. Following

reconstitution, the liposomes were processed using the same procedure as the non-lyophilized formulation.

Gentamicin concentration was quantified using ninhydrin spectrofluorimetric assay. The reaction mixture was incubated at 75°C for 10 min, immediately cooled in an ice bath for 10 min, then analyzed using a spectrofluorometer at a wavelength of 405 nm. Gentamicin concentration was calculated from a previously established calibration curve prepared under identical experimental conditions. Encapsulation efficiency was determined by direct quantification of the drug extracted from the liposomal pellet and was calculated using the following equation [\(Eq.1\)](#):

$$y = 0.2024x - 0.0064 \quad (Eq.1)$$

Square linear regression coefficient achieved for GS in PBS solution was $R^2 = 0.9429$

A calibration curve was prepared to enable quantitative determination of gentamicin concentration using the ninhydrin spectrofluorimetric assay. Gentamicin sulfate standard solutions were prepared in PBS of pH 7.4 over a concentration range of 0.007–1 mg/mL ([supplementary data Figure S1](#)).

The EE % represents the percentage of drug encapsulated into the liposomes compared to the total amount of drug initially used in the formulation ([Eq.2](#)).

$$EE\% = \frac{\text{Mass of encapsulated drug}}{\text{Total mass of drug added in the formulation}} \times 100 \quad (Eq.2)$$

3.2.4. Physicochemical Characterization

Characterization of both placebo and GS-loaded liposomes was initially performed at the University of Pavia using Dynamic Light Scattering (DLS) Nicomp 380 ZLS Particle Sizing System (Particle Sizing Systems, CA, USA), before and after lyophilization, to assess the effect of the lyophilization process on the formulations size and PDI. Following shipment of the lyophilized formulations to TTMI, further characterization was performed to evaluate whether shipment affected their physicochemical properties. At TTMI, the reconstituted liposomes were

characterized using three complementary analytical instruments: Dynamic Light Scattering (DLS) (Malvern Instruments, Malvern Panalytical, Zetasizer, UK), Nanoparticle Tracking Analysis (NTA) NanoSight PRO (Malvern Panalytical, UK) and VideoDrop (Myriade, Paris, FR). These instruments provided complementary information on particle size range, size distribution, and particle concentration, allowing a more comprehensive assessment of physicochemical characteristics of the liposomal formulations, exact technique of each instrument is further explained in the [Supplementary Data](#). Following shipment to TTMI (Dublin, Ireland), the lyophilized powders were reconstituted in 1 mL of PBS (pH 7.4) to restore their original formulation volume and subjected to magnetic stirring at 300 rpm for 30 min to ensure complete and homogeneous reconstitution. All characterization measurements were performed both before and after filtration through a 0.22 μm membrane.

3.2.5. Electroporation (EP) of lyophilized liposomes

Electroporation was conducted at TTMI (Dublin, Ireland) using the ePORE[®] electroporation generator (Mirai Medical, Galway, Ireland). Electroporation was initially performed on both the 3:1 and 5:1 GS-loaded formulations for the preliminary formulation-selection study described below ([section 3.2.6](#)). Following selection of the 5:1 formulation, the same electroporation conditions were applied to the 5:1 formulation throughout all subsequent experiments. Prior to electroporation (EP), the lyophilized liposomes stored at 4°C prior to rehydration were prepared and reconstituted with 1ml of PBS at pH 7.4. The resulting 1 ml suspension was divided into ten aliquots of 100 μL each and transferred into sterile electroporation cuvettes with a 2 mm electrode gap. Electroporation was then performed using four different voltage conditions: 0 V (control), 160 V, 200 V, and 250 V. The 0 V group was processed identically to the electroporated samples but without the application of an electric pulse, serving as the experimental control. Parameters used include, number of pulses (8), pulse length (100 μs), frequency (500 Hz); unipolar positive high-voltage pulses. These parameters are consistent with the protocol suggested by standard operator procedures (ESOPPE) for EP use in clinic [[11](#), [16](#)].

Following electroporation, the liposomal suspensions were recovered from the cuvettes and used for subsequent analyses according to the experimental design. Depending on the

experiment, the electroporated samples were subjected to physicochemical characterization and *in vitro* release studies.

3.2.6. Preliminary *in vitro* release study and formulation selection

Although the 5:1 formulation had previously undergone physicochemical characterization and encapsulation-efficiency assessment during formulation development in Pavia, as described in [section 3.2.1](#) a comparative *in vitro* release study was subsequently performed at TTMI (Dublin, Ireland). This was carried out to determine which of the two GS-loaded formulations (3:1 or 5:1) would be carried forward for the subsequent experimental studies. Following electroporation, both formulations were subjected to the same identical release conditions using PBS at physiological pH (7.4), using the dialysis procedure described below in [section 3.2.6.1](#). Based on the resulting release profiles, 5:1 formulation was selected for subsequent experimental investigations. Accordingly, the extended *in vitro* release studies under different media and pH conditions, as well as storage and stability studies, were conducted using the 5:1 GS-loaded formulation.

3.2.6.1. *In vitro* drug release experiments of the selected formulation

The *in vitro* release profile of gentamicin from the lyophilized liposomal formulations was evaluated using the dialysis membrane method with a molecular weight cut-off (MWCO) of 12–14 kDa. Following electroporation as reported in [section 3.2.5](#), each 100 µL liposome aliquot was divided into two 50 µL portions to obtain two experimental replicates while maintaining a gentamicin concentration relevant to the intended clinical application. The volume of each 50 µL sample was then adjusted to 1 mL using PBS to prepare the samples for *in vitro* release testing and transferred to the dialysis membrane. The dialysis bags were immersed in 6 mL of the corresponding release medium contained in sterile tubes and incubated at 37°C under continuous magnetic stirring at 300 rpm to maintain sink conditions throughout the experiment. At predetermined time intervals (5, 15, 30 min, 1, 2, 3, 6, 24, 48, 72, 144 hours), 1 mL of the external released medium was withdrawn for analysis and immediately replaced with an equal volume of fresh pre-warmed release medium to maintain a constant volume and sink conditions.

For each replicate, five experimental conditions were prepared: liposomes treated with 0 V (control), 160 V, 200 V, and 250 V, in addition to a sample treated with 2% Triton X-100. Triton X-100 was used to completely disrupt the liposomal membrane and release the encapsulated gentamicin, thereby providing the maximum releasable drug content. This value was subsequently used as the amount of drug encapsulated in liposomes reported in (Eq.3) for calculating the cumulative percentage of gentamicin released at each sampling time point during the *in vitro* release study using equation (Eq.3) The procedure described above was applied throughout all subsequent experiments.

Depending on the experimental objective, reconstituted liposomal formulations were evaluated using different release media, including PBS, physiological saline (0.9% NaCl), and DMEM supplemented with human serum albumin (HSA; BioReagent, ≥96% purity, A1653; Sigma-Aldrich, St. Louis, MO, USA). The effect of environmental pH on liposomal behavior and drug release was investigated under physiological conditions (pH 7.4) and mildly acidic conditions (pH 6.5 and 5.5). For the experiments involving DMEM supplemented with HSA, the liposomal formulation was dispersed in the supplemented culture medium and placed inside the dialysis membrane, while PBS adjusted to either pH 7.4 or pH 5.5 was used as the external release medium.

For each release media a calibration curve was performed to calculate the gentamicin concentration released at each timepoint (Supplementary data S2-S5).

$$\% \text{ Drug release} = \frac{\text{Cumulative amount of drug released at time (t)}}{\text{Amount of drug encapsulated in liposome}} \times 100 \quad (\text{Eq. 3})$$

3.2.7. Stability study design

The long-term stability of both placebo and gentamicin-loaded liposomes was carried out following lyophilization to determine the influence of storage temperature and storage duration on the physicochemical properties of the formulations.

For both placebo and gentamicin- loaded liposomes, eight independent batches of lyophilized formulation were prepared. The samples were stored at four different temperatures: 4°C - refrigerated, room temperature RT (23-25°C), 37°C incubated with 5% CO₂, and –20°C in the

freezer. Two batches were assigned to each storage condition to provide duplicate samples for analysis. Each placebo batch contained 5 mg of lyophilized powder, while gentamicin-loaded liposomes contained 25 mg of lyophilized powder.

The stability of both formulations was assessed after 1 week, 1 month and 3 months duration of storage. At each predetermined time point, the corresponding samples were reconstituted in 1 ml PBS and characterized to evaluate changes in particle size, size distribution, and particle concentration. Physicochemical characterization was performed using three complementary analytical instruments, Malvern DLS, NTA PRO and VideoDrop. For each sample, measurements were run before and after filtration. Filtration was carried out using a sterile 0.22 µm membrane filter to remove larger particles and aggregates. The same reconstitution, filtration, and characterization procedures were applied to both placebo and gentamicin-loaded liposomes at every storage temperature and time point.

In addition to physicochemical characterization, the gentamicin-loaded liposomes underwent quantitative analysis of the encapsulated gentamicin at each storage temperature and time point. Gentamicin content was determined using the calibration curve ([Eq.1](#)) and previously described analytical method in [section 3.2.3](#), allowing the percentage of drug retained within the formulation to be calculated throughout the storage time using equation ([Eq.4](#)). The remaining gentamicin content was subsequently used to construct the degradation profile of the formulation under each storage condition over time and to evaluate the influence of storage temperature on the long-term chemical stability of the encapsulated drug.

$$\% \text{ Retention} = \frac{\text{GS concentration at time } (t)}{\text{GS concentration at Day 0}} \times 100 \quad (\text{Eq.4})$$

3.2.8. Statistical Analysis

Data analysis, graphical representation and statistical analyses were performed using GraphPad Prism version 11.0 (GraphPad Software, San Diego, CA, USA).

Data normality was assessed using the Shapiro–Wilk normality test. Comparisons between two experimental groups were performed using either an unpaired Student's t-test or a paired

Student's t-test, depending on the experimental design. For comparisons involving more than two groups, statistical significance was evaluated using one-way analysis of variance (ANOVA). Dunnett's multiple comparison test was used when each experimental group was compared with a single control group, whereas Tukey's multiple comparison test was applied for pairwise comparisons among all experimental groups. For experiments involving two independent variables, such as storage temperature and storage time or electroporation voltage and release medium, two-way ANOVA followed by the appropriate multiple comparison test was performed when applicable.

Results are presented as the mean $\pm$ standard deviation (SD). Unless otherwise stated, all experiments were performed in triplicate ($n = 3$). A p -value < 0.05 was considered statistically significant.

4. Results

4.1. Lyophilization of the liposomes

The lyophilization process produced ten dry cake batches, with yields ranging from 255.4 to 290.1 mg, as presented in [Table.2](#) and extensively reported in the [Supplementary Data](#).

4.2. Encapsulation Efficiency (EE %) of 5:1 GS liposome formulation

Following formulation of the 5:1 GS liposomes in Pavia as reported in [section 3.2.1](#). The EE % was determined by directly quantifying the amount of gentamicin encapsulated inside the liposomes. This amount was determined from the equation obtained by the calibration curve shown in [section 3.2.3](#) based on the intensity of fluorescence obtained following the measurement with Spectro-fluorimetry. Before lyophilization, the mean encapsulation efficiency was $18.43 \pm 2.80\%$, whereas following lyophilization and reconstitution, an encapsulation efficiency of $17.09 \pm 2.26\%$ as reported in [Figure 3](#). No statistically significant difference in encapsulation efficiency was observed before and after lyophilization ($p > 0.05$), indicating that the freeze-drying and reconstitution processes did not significantly compromise gentamicin retention within the liposomal formulation.

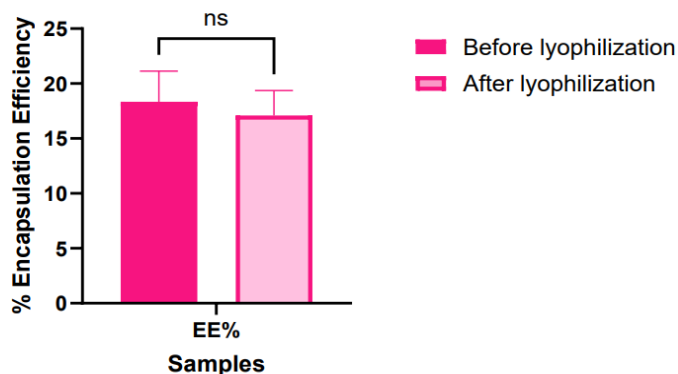


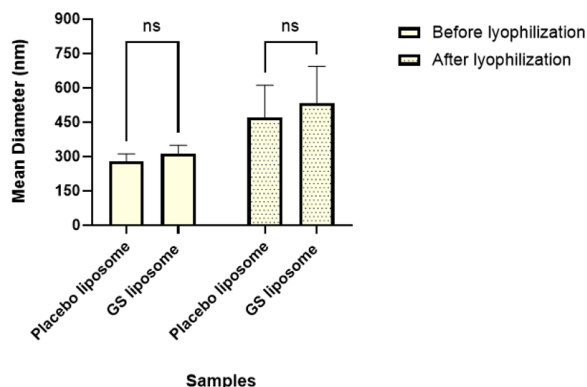
Figure 3. Encapsulation efficiency (%) of 5:1 GS-loaded liposomes before and after lyophilization following preparation and lyophilization in Pavia (ns: not significant, p-value > 0.05).

4.3. Liposome characterization before and after lyophilization

Particle size variation of both placebo (PLC) and GS- loaded liposomes after freeze-drying process was initially evaluated using DLS Nicomp 380 ZLS Particle Sizing System (Particle Sizing Systems, CA, USA) in Pavia. Mean particle diameter was measured before freeze-drying and after reconstitution of the lyophilized formulations. The results are presented in [Figure 4](#).

For the placebo liposomes, the mean particle diameter increased from 254.0 ± 85.5 nm and PDI 0.161 ± 0.020 before lyophilization to approximately 370.0 ± 64.8 nm and PDI 0.439 ± 0.012 after reconstitution ([Figure 4](#)). A similar trend was observed for the gentamicin-loaded liposomes, where the mean particle diameter increased from approximately 288.0 ± 118.6 nm and PDI 0.299 ± 0.023 before lyophilization to approximately 360.0 ± 153.0 nm and PDI 0.695 ± 0.050 following reconstitution ([Figure 4](#)). Compared with the placebo formulation, gentamicin-loaded liposomes exhibited slightly higher mean particle diameter and PDI values both before and after lyophilization. Following reconstitution, the increase in both particle size and PDI observed for PLC and GS-loaded formulations according to DLS measurement was not statistically significant ($p > 0.05$). The graphical panels are reported in [supplementary data \(Figures S6-S7\)](#).

Mean Diameter Variation of PLC and GS liposome after lyophilization using DLS



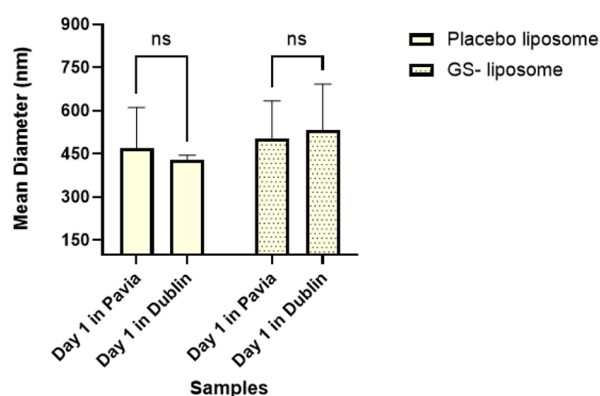
Placebo Liposome	Before lyophilization	After lyophilization
PDI	0.161 ± 0.02	0.439 ± 0.012

GS- Liposome	Before lyophilization	After lyophilization
PDI	0.299 ± 0.023	0.695 ± 0.05

Figure 4. Mean particle diameter and PDI of PLC and GS-loaded liposomes before and after lyophilization as determined by DLS instrument in Pavia. Measurements were performed following reconstitution of the lyophilized formulations with PBS. (ns: not statistically significant, $p > 0.05$). Data are presented as mean $\pm$ SD.

To assess whether the shipment of the lyophilized formulations from Pavia to Dublin affected their physical characteristics, the mean particle diameter of PLC and GS-loaded liposomes was compared with DLS measurements carried out at TTMI (Dublin, Ireland). No statistically significant differences were observed between the measurements run in Pavia and those obtained in TTMI (Dublin, Ireland). This applied to both PLC or GS liposomes ($p > 0.05$) as reported in [Figure 5](#). These results indicated that the shipment did not significantly affected the mean diameter of formulation, supporting the physical stability of the lyophilized liposomes. The DLS extensive characterization is reported in graphical panel format in the [supplementary data Figure 5](#). To determine the most appropriate particle-size parameter for subsequent analyses, the mean and modal diameters of PLC and GS-loaded liposomes were compared following lyophilization. No statistically significant differences were observed between the recorded mean and modal diameter for both formulation ($p > 0.05$); full graphical panel is reported in the [supplementary data \(Figure 9\)](#). Therefore, mean diameter was selected as representative of particle-size parameter and was used for all subsequent analyses and comparisons, since no critical difference was observed between these two size parameters.

Mean Diameter Variation of PLC and GS-liposomes after lyophilization using DLS



Placebo Liposome	Day 1 in Pavia	Day 1 in Dublin
PDI	0.439 ± 0.012	0.505 ± 0.06

GS- Liposome	Day 1 in Pavia	Day 1 in Dublin
PDI	0.695 ± 0.05	0.582 ± 0.103

Figure 5. Mean diameter and PDI of PLC and GS-loaded liposomes measured by DLS in Pavia before shipment and in TTMI (Dublin, Ireland) after shipment. No statistically significant differences were observed between the two locations for either formulation (ns, $p > 0.05$). Data are presented as mean $\pm$ SD.

Further characterization of the reconstituted lyophilized formulations was performed at TTMI (Dublin, Ireland) using three complementary instruments: DLS, NTA-PRO and VideoDrop. Particle size was evaluated both before and after filtration through a 0.22 μ m membrane, as presented in [Figure 6](#).

Before filtration, NTA-PRO and VideoDrop yielded comparable particle size measurements for both PLC and GS-loaded liposomes. For PLC liposomes, the mean particle diameters determined by NTA-PRO and VideoDrop were 189.0 ± 177.0 nm and 168.0 ± 68.0 nm, respectively. Similarly, for GS liposomes, mean particle diameters of 166.0 ± 40.4 nm and 200.0 ± 39.1 nm were obtained using NTA-PRO and Videodrop, respectively ([Figure 6](#)). In contrast, DLS reported statistically significant (p -value < 0.0001 and p -value < 0.05) larger mean particle diameters before filtration, reaching 420.0 ± 8.5 nm for placebo liposomes and 427.0 ± 12.7 nm for GS liposomes as reported in [Figure 6](#). Following filtration through a 0.22 μ m membrane, greater agreement was observed among the three analytical equipment. For placebo liposomes, the mean particle diameters determined by DLS, NTA-PRO and VideoDrop were 110.3 ± 44.8 nm, 125.0 ± 86.0 nm and 177.0 ± 173.0 nm respectively. For GS liposomes, the corresponding mean diameters were 148.0 ± 39.1 nm, 127.0 ± 24.2 nm, and 160.0 ± 19.2 nm respectively.

Overall, the greatest differences among the analytical instruments were observed in the unfiltered samples, where DLS reported substantially larger particle sizes than the NTA-PRO and

the VideoDrop instrument. The graphical panel showing these differences is extensively reported in the [supplementary data \(Figures S10-S11\)](#). Following filtration, the particle size measurements greater agreement among the three analytical instruments. These findings emphasized the importance of complementing DLS measurements with particle-based techniques such as NTA-PRO and VideoDrop, particularly when evaluating heterogeneous liposomal populations rather than relying exclusively on one single technique such as DLS for the physicochemical characterization.

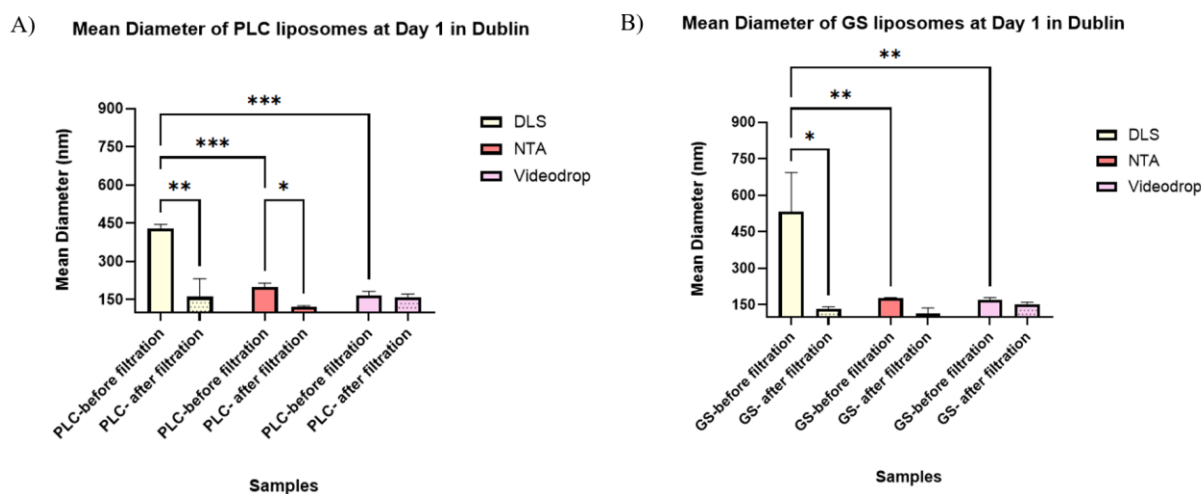


Figure 6. Mean particle diameter (nm) of (A) lyophilized PLC liposomes and (B) lyophilized GS-liposomes before and after filtration through a 0.22 µm membrane filter using DLS, NTA-PRO and VideoDrop instruments.

4.4. *In vitro* drug release study

4.4.1. Preliminary *in vitro* release study and formulation selection

Gentamicin release from the liposomal formulations was quantified using the ninhydrin-based colorimetric assay described in [section 3.2.3](#). At each sampling time point, the amount of gentamicin released into the external medium was determined using a calibration curve prepared under the corresponding experimental conditions ([Supplementary data S2-S5](#)). The results were expressed as cumulative gentamicin release (%) relative to the total amount of gentamicin encapsulated in the liposomal formulation. The cumulative gentamicin release profiles of the 5:1 and 3:1 formulation was compared over 144 h, with particular attention to the initial 6 h following electroporation. Overall, the 5:1 formulation exhibited a more pronounced

early drug release than the 3:1 formulation, particularly at 0, 160, and 250 V. At 6 h, the 5:1 formulation released approximately 32%, 58%, 45%, and 88% of the encapsulated drug at 0, 160, 200, and 250 V, respectively as reported in [Figure 7](#). In comparison, the 3:1 formulation showed approximately 27%, 26%, 46%, and 64% release under the same respective conditions. The difference was particularly evident at 160 V and 250 V, where the 5:1 formulation showed substantially greater release during the first 6 h.

Over the extended 144 h period, both formulations showed progressive drug release, although differences between the formulations became less pronounced. Thus, the main advantage of the 5:1 formulation was observed during the early release phase, rather than at the final time point. Based on its greater initial drug release, particularly following electroporation at 160 and 250 V, the 5:1 formulation was selected for the subsequent release and stability studies.

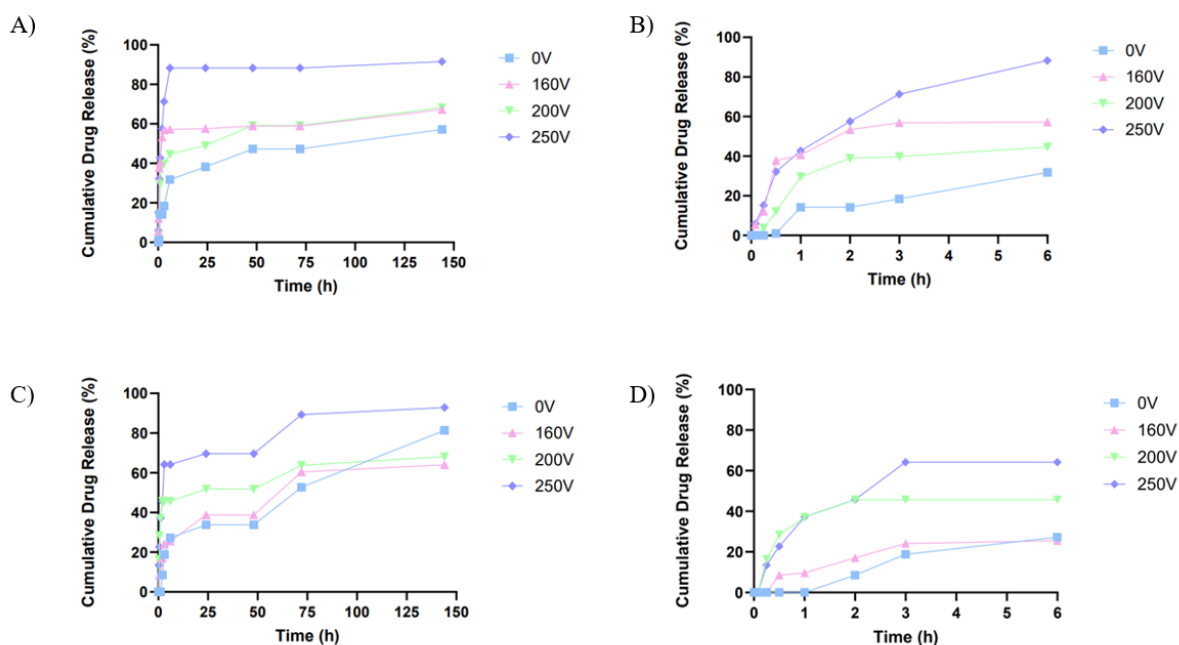


Figure 7. In vitro cumulative release profiles of gentamicin from lyophilized liposomes following electroporation at 0, 160, 200, and 250 V in PBS. (A) 5:1 liposomal formulation release over 144 h; (B) 5:1 liposomal formulation release during the first 6 h; (C) 3:1 liposomal formulation release over 144 h; (D) 3:1 liposomal formulation release during the first 6 h.

4.4.2. *In vitro* release study of 5:1 Liposomal Formulations

The *in vitro* release behavior of gentamicin-loaded liposomes was evaluated under different release conditions as reported in [section 2.2.7](#). Drug release was monitored up to 144 hours, with the first 6 hours examined to evaluate the early release phase. The cumulative release profiles measured by microplate spectrophotometer are presented in [Figure 8](#) and [Figure 9](#).

At physiological pH (7.4), gentamicin release increased progressively over the 144-h experimental period for all electroporation conditions ([Figure 8.A](#)). Differences among the voltage conditions were particularly evident during the early phase of the experiment ([Figure 8.B](#)). The liposomes electroporated at 160 V, 200 V and 250 V exhibited more rapid initial release starting from the first hour in comparison to the non-electroporated liposomes at 0 V (negative control for EP). Following this initial phase, cumulative release continued to increase under all conditions. This rapid release observed following electroporation may be attributed to the formation of reversible pores, facilitating the diffusion of the hydrophilic gentamicin drug. By the end of the 144-h experiment, the electroporated liposomes from 160 V to 250 V exhibited approximately similar release profile as the 0V formulation. This pattern observed support that the pores created seal after a short period of time. At pH 6.5, a voltage-dependent difference in the early release profile was also observed ([Figure 8. D](#)). The 250 V condition showed the more prominent initial release as the case at physiologic pH, reaching approximately 20% within the first 30 min and approximately 36% after 6 h. Over the extended 144-h period ([Figure 8. C](#)), cumulative gentamicin release was significantly higher for the electroporated formulations compared to physiologic pH ([Figure 8. D](#)).

The release profile at pH 5.5 similarly showed differences among the electroporation conditions. The most pronounced early release observed following electroporation at 250 V. In contrast, 160 V and 200 V had a similar initial release profile as the 0V formulation after 6 h. This trend would lead to hypothesis that at 250V the rearrangement of the lipids in the bilayer membrane occurred following electroporation ([Figure 8. F](#)). After 144 h, the 200V and the 250V formulations exhibited the highest cumulative drug release ([Figure 8. E](#)). Overall, the release profiles demonstrated

differences in cumulative gentamicin release according to both environmental pH and applied electroporation voltage, with the greatest early release observed following electroporation at 250 V, particularly under acidic conditions.

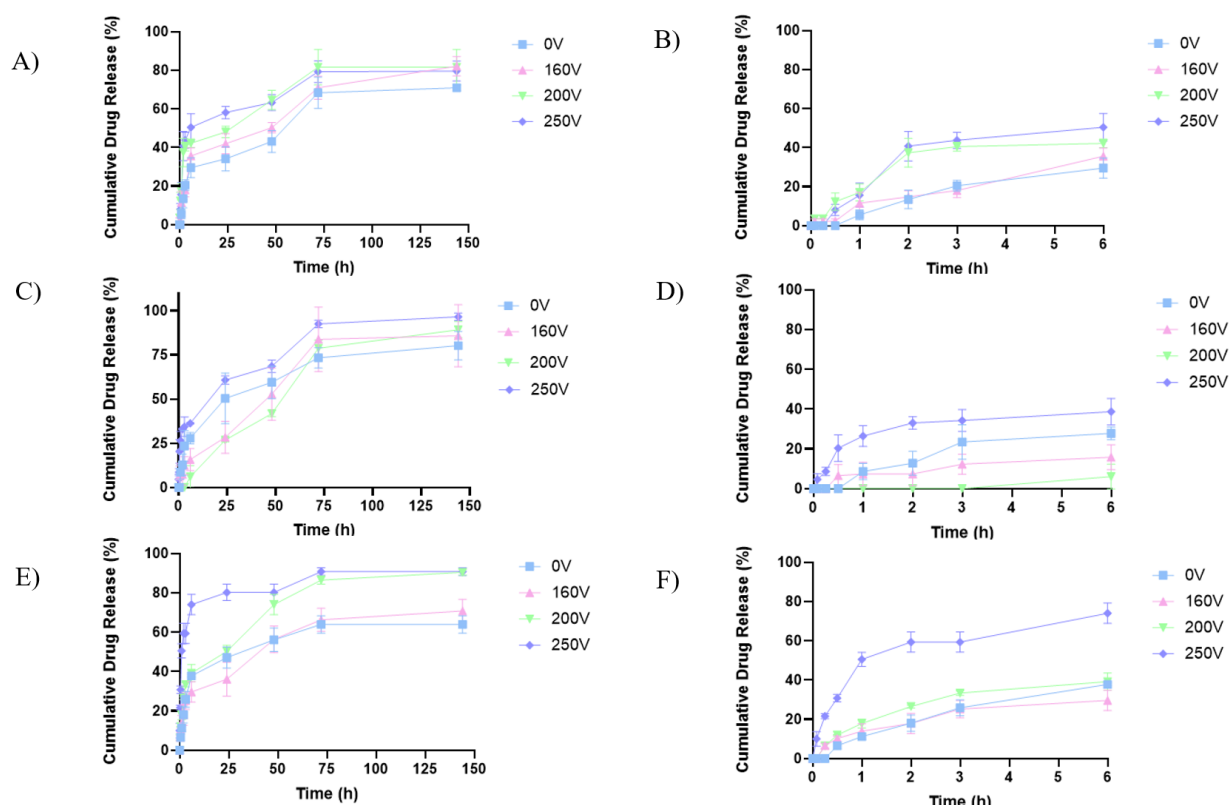


Figure 8. In vitro cumulative release profiles of gentamicin from lyophilized liposomes following electroporation at 0, 160, 200, and 250 V in PBS at different pH conditions. (A) PBS pH 7.4 over 144 h; (B) PBS pH 7.4 during the first 6 h; (C) PBS pH 6.5 over 144 h; (D) PBS pH 6.5 during the first 6 h; (E) PBS pH 5.5 over 144 h; and (F) PBS pH 5.5 during the first 6 h.

Comparison of the different release media demonstrated clear differences in both the rate and extent of gentamicin release ([Figure 9](#)).

During the first 6 h, saline produced a progressive release across all electroporation voltages ([Figure 9. B](#)), with 200 V and 250V showing the highest cumulative release values. In contrast, culture medium supplemented with HSA showed a stronger dependence on both pH and applied voltage. At pH 7.4, the electroporated formulations produced greater early release than the non-electroporated control ([Figure 9.D](#)), whereas at pH 5.5, the 250 V condition produced the most pronounced burst release, reaching approximately 80% within the first 3–6 h as shown in [Figure 9.F](#). At the later time points, saline produced nearly complete release at 200V and 250 V, reaching

approximately 97–100% after 144 h ([Figure 9. A](#)). Culture medium supplemented with HSA at pH 7.4 similarly resulted in high final release at 200 and 250 V, reaching approximately 95% ([Figure 9. C](#)). At pH 5.5, the electroporated formulations had a similar release profile as the 0V control after 144h ([Figure 9.E](#)).

Overall, the release profiles did not differ significantly upon changing the composition and pH of the release medium, while the effect of electroporation was also dependent on the applied voltage. The strongest early release was observed at 250 V in all release culture media, and the release was generally more gradual.

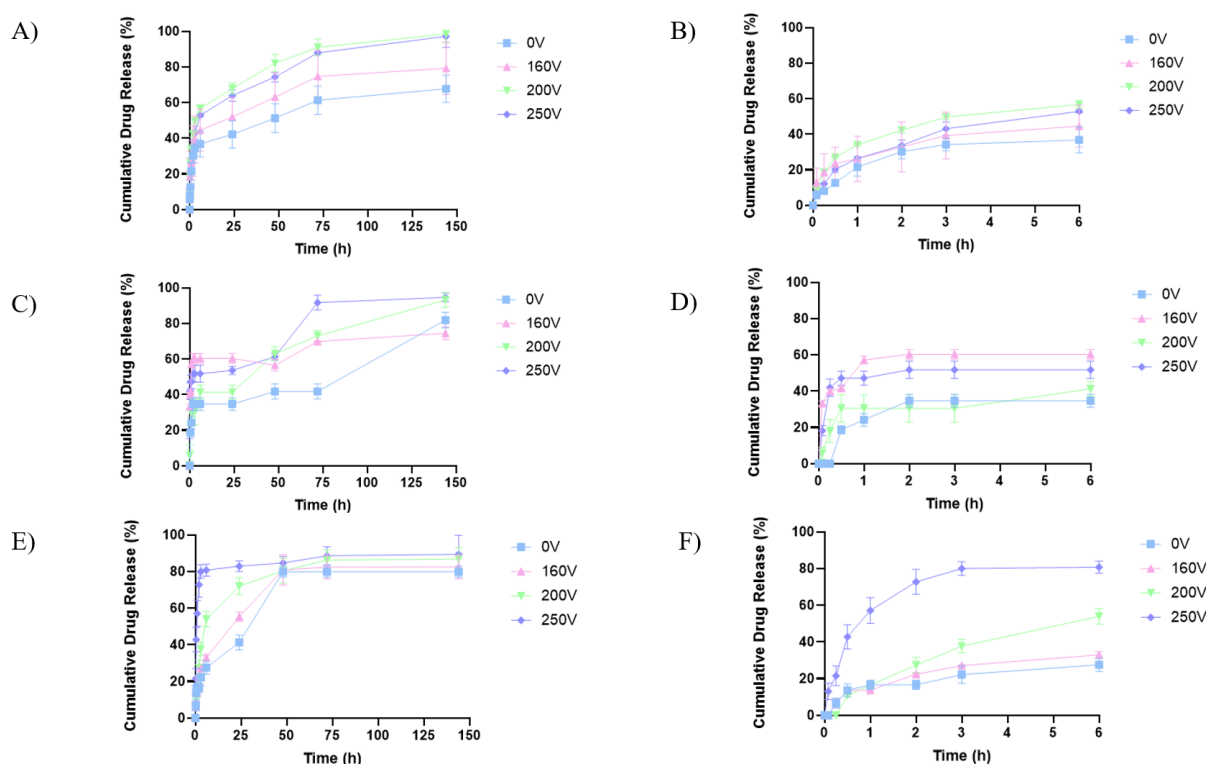


Figure 9. In vitro cumulative release profiles of gentamicin-loaded liposomes following electroporation at 0, 160, 200, and 250 V under different release conditions. (A) Saline over 144 h; (B) saline during the first 6 h; (C) culture medium supplemented with HSA at pH 7.4 over 144 h; (D) culture medium supplemented with HSA at pH 7.4 during the first 6 h; (E) culture medium supplemented with HSA at pH 5.5 over 144 h; and (F) culture medium supplemented with HSA at pH 5.5 during the first 6 h.

4.5. Electroporation and physiochemical characterization following EP

The effect of electroporation on the particle size of gentamicin-loaded (GS) liposomes was evaluated at four different applied voltages (0, 160, 200, and 250 V). Particle size was determined

using DLS, NTA-PRO and VideoDrop instrument. These were carried out before and after filtration through a 0.22 μm membrane ([Figure 10](#)).

Before filtration, the mean particle diameter remained relatively stable across the different electroporation conditions ([Figure 10.A](#)). DLS reported the largest particle sizes, with mean diameters ranging approximately between 273.0 ± 34.0 nm and 300.0 ± 187.9 nm across the 0–250 V conditions. VideoDrop measurements remained approximately between 154 ± 9.19 nm and 188.0 ± 67.9 nm, while NTA reported mean particle diameters of approximately 150.0 ± 19.8 nm to 173.0 ± 34.6 nm. No statistically significant differences were observed among the investigated conditions before filtration. Overall, increasing the voltage applied from 0V to 250 V did not result in an observable change in the mean particle diameter of the unfiltered GS-loaded liposomes.

Following filtration through a 0.22 μm membrane, differences in the particle sizes reported by the three analytical techniques became more pronounced at higher electroporation voltages ([Figure 10.B](#)). At 0 V, DLS reported a mean particle diameter of approximately 296.0 ± 65.5 nm, compared with approximately 125.0 ± 7.1 nm and 138.0 ± 34.6 nm using NTA-PRO and VideoDrop, respectively. At 160 V, the corresponding mean diameters were approximately 281.0 ± 11.5 nm, 129.0 nm and 182.0 ± 56.6 nm for DLS, NTA-PRO and VideoDrop respectively. A further increase in the DLS-measured particle diameter was observed at higher voltages, reaching approximately 381.0 ± 103.0 nm at 200 V and 454 ± 24.0 nm at 250 V, but this increase is not statistically significant. In contrast, NTA-PRO and VideoDrop measurements remained comparatively stable, with mean particle diameters predominantly below 200 nm.

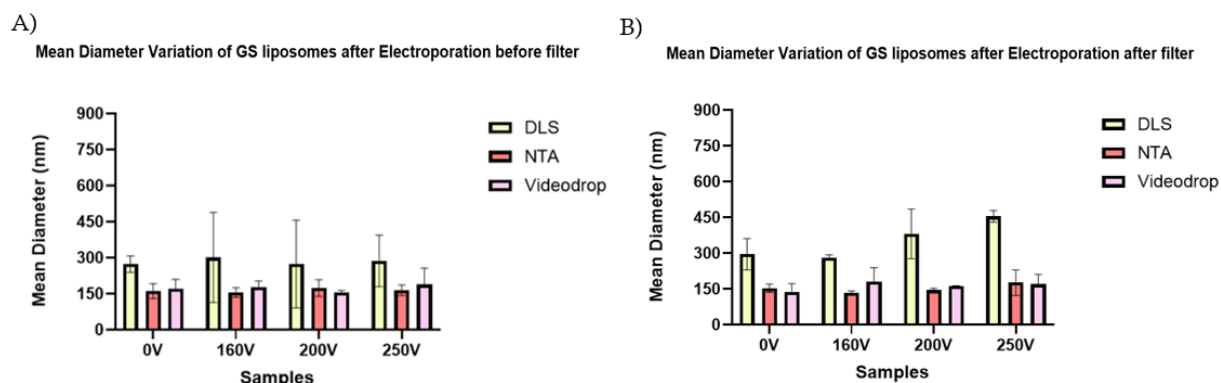


Figure 10. Mean Diameter Variation of GS-liposomes after electroporation at different voltages 0V, 160V, 200V, and 250 V as determined by DLS, NTA-PRO and VideoDrop. (A) before filtration and (B) after filtration through a 0.22 µm membrane. Data are presented as mean ± SD. PDI results are represented in the [supplementary data Table 3](#).

4.6. Stability study

The stability of lyophilized placebo liposomes was evaluated following storage at 4°C, RT (23-25°C), 37°C incubated with 5% CO₂, and -20°C as reported in [section 2.2.8](#). Samples were stored in closed Eppendorf tubes additionally sealed with paraffin to minimize exposure to environmental moisture. Although relative humidity differed among the storage environments and was not specifically controlled, the additional sealing was intended to limit moisture exchange; therefore, temperature was considered the principal storage variable evaluated in this study.

4.6.1. Stability of placebo liposomes

After one week of storage, differences in particle size were observed among the four storage conditions, particularly in the measurements obtained before filtration as reported in [Figure 11.A](#). DLS measurements showed a substantial increase in particle size following storage under most tested conditions compared to the freshly prepared placebo liposomes. The mean particle size at Day 0 was 418.0 ± 99.0 nm, which increased to 516.0 ± 173.0 nm, 636.0 ± 120.0 nm, 700.0 ± 97.0 nm, and 544.0 ± 213.0 nm for samples stored at 4°C, room temperature, 37°C, and -20°C, respectively, as shown in [Figure 11.A](#). The only statistically significant increase in particle size was observed in samples stored at 37°C, while samples stored at 4°C exhibited the lowest average increase. NTA-PRO and VideoDrop showed closer result dataset. In contrast, NTA-PRO and VideoDrop reported considerably smaller particle sizes than DLS before filtration, with mean

diameters predominantly below approximately 250 nm across the different storage conditions as reported in [Figure 11.A](#). The average particle-size trend measured by DLS was substantially higher than those obtained with the particle-tracking techniques as reported in [supplementary data \(Figure S12\)](#), whereas the NTA-PRO and VideoDrop measurements were closely aligned. This difference suggests the presence of a larger-particle population in the unfiltered samples, which had a greater influence on the DLS measurements. Following filtration through a 0.22 μm membrane, a marked reduction in the particle sizes measured by DLS was observed, and greater agreement was obtained among DLS, NTA-PRO and VideoDrop ([Figure 11.B](#)). Across the four storage conditions, the mean particle diameters after filtration were predominantly within the expected range of approximately of 100–190 nm.

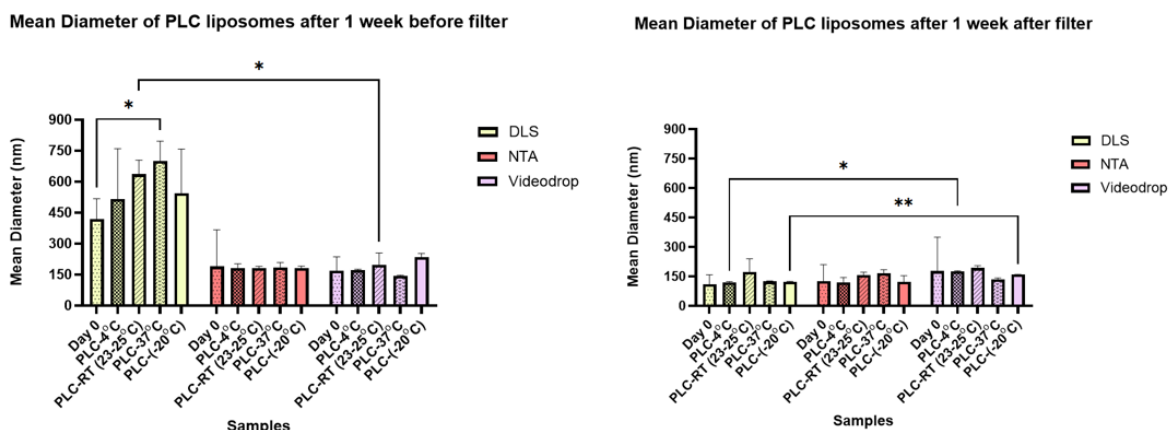


Figure 11. Particle mean size (nm) of placebo liposomes (A) before filtration and (B) after filtration relative to Day 0 following one week storage at 4°C, room temperature RT (23–25°C), 37°C incubated with 5% CO₂ and - 20°C. Data are presented as mean $\pm$ SD. * $p < 0.05$.

The physicochemical stability of placebo liposomes was further evaluated after one month of storage at 4°C, room temperature (RT), 37°C, and –20°C. Particle size was determined using DLS, NTA and VideoDrop both before and after filtration through a 0.22 μm membrane ([Figure 12.A](#)).

DLS measurements showed a Day 0 mean particle diameter of approximately 418.0 $\pm$ 99.2 nm. Following one month of storage, the mean particle size increased under all storage conditions, although magnitude depended on temperature. The largest statistically significant increase was observed for samples stored at 37°C, where the mean particle diameter reached approximately 516.3 $\pm$ 92.5 nm [Figure 12.A](#). NTA-PRO measurements demonstrated smaller changes in particle

size than DLS. The Day 0 mean diameter was approximately 189.0 ± 177.0 nm, the mean diameters were predominantly below approximately 200 nm across the different storage conditions as shown in [Figure 12.A](#). Similarly, VideoDrop measurements showed modest variations in particle size compared with DLS. The mean particle diameter increased from approximately 168.0 ± 68.0 nm at Day 0 to 230.0 ± 64.3 nm after storage at 4°C , 238.0 ± 55.1 nm at room temperature, and 200.0 ± 38.9 nm following storage at 37°C as reported in [Figure 12.A](#). Samples stored at -20°C exhibited a mean diameter of approximately 200 ± 21.21 nm, remaining closest to the freshly prepared formulation.

Following filtration, differences among the three characterization techniques were markedly reduced ([Figure 12.B](#)). DLS measurements decreased to approximately 100–175 nm, while NTA and VideoDrop similarly reported particle sizes predominantly within the range of approximately 100–190 nm. Thus, as observed after one week of storage, filtration resulted in greater agreement among DLS, NTA-PRO and VideoDrop measurements.

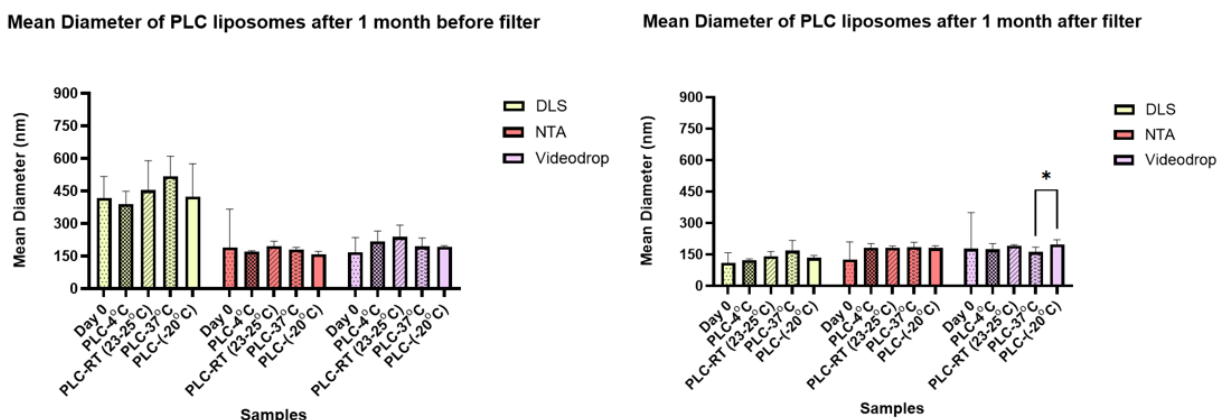


Figure 12. Particle mean size (nm) of placebo liposomes (A) before filtration and (B) after filtration relative to Day 0 following one month storage at 4°C , room temperature RT ($23-25^{\circ}\text{C}$), 37°C incubated with 5% CO_2 and -20°C . Data are presented as mean $\pm$ SD. * $p < 0.05$.

After three months of storage and before filtration ([Figure 13.A](#)), DLS reported substantially larger mean particle diameters than NTA-PRO and VideoDrop. The mean DLS diameter increased from 418.0 ± 99.2 nm at Day 0 to 462.9 ± 146.4 nm at 4°C , 513.8 ± 91.2 nm at RT, and 607.2 ± 33.7 nm at 37°C , whereas the sample stored at -20°C showed a mean diameter of 454.5 ± 101.2 nm. The largest mean diameter was therefore observed following storage at 37°C . A statistically

significant difference was observed between the 37°C and –20°C DLS measurements ($p < 0.05$), as indicated in [Figure 13.A](#).

In contrast, VideoDrop reported considerably smaller mean particle diameters of 168.00 ± 0.00 nm at Day 0, 183.00 ± 16.97 nm at 4°C, 237.50 ± 85.56 nm at RT, 190.50 ± 17.68 nm at 37°C, and 206.50 ± 7.78 nm at –20°C. No statistically significant differences among the VideoDrop recording over the storage conditions are indicated in the figure. Whereas the NTA-PRO similarly reported smaller particle sizes than DLS, with a mean diameter of 189.0 ± 0.0 nm at Day 0, increasing to 258.0 ± 7.1 nm at 4°C and 254.0 ± 8.5 nm at RT, while values of 226.0 ± 1.4 nm at 37°C and 241.0 ± 28.3 nm at –20°C were observed. A statistically significant difference ($p < 0.05$) was observed between Day 0 liposomes and liposomes stored at 37°C indicated in [Figure 13.A](#). Overall, before filtration, the most pronounced increase in particle size was observed by DLS, particularly following storage at 37°C.

Following filtration ([Figure 13.B](#)), the mean particle sizes were substantially reduced, and greater agreement was observed among the three analytical techniques. DLS measurements decreased to approximately 100–175 nm. Conversely, NTA-PRO and VideoDrop similarly reported particle sizes predominantly within the range of approximately 100–190 nm.

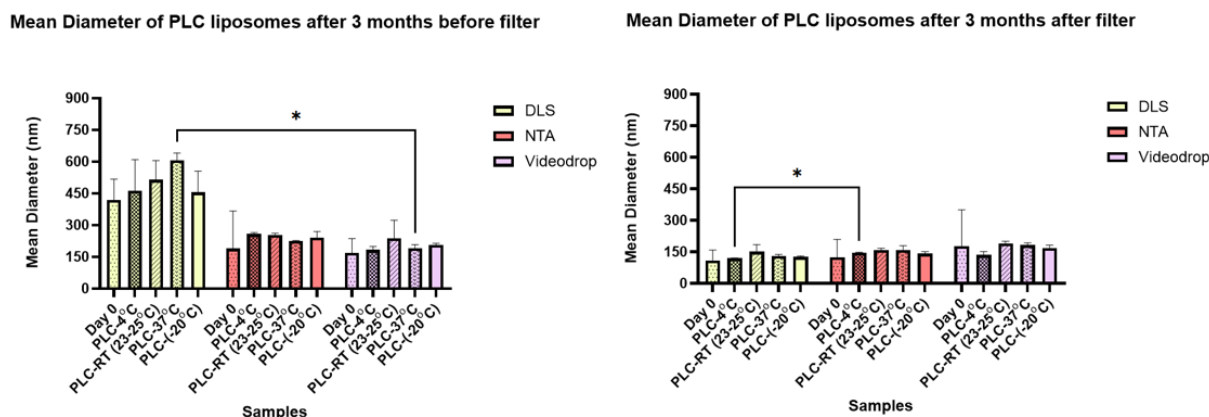


Figure 13. Particle mean size (nm) of placebo liposomes (A) before filtration and (B) after filtration relative to Day 0 following three months of storage at 4°C, room temperature RT (23–25°C), 37°C incubated with 5% CO₂ and –20°C. Data are presented as mean $\pm$ SD. * $p < 0.05$.

4.6.1.1. Comparison of particle retention during storage

To evaluate the effect of storage on placebo liposomes physical stability, the particle concentration retention was determined after three months of storage at 4°C, RT (23-25°C), 37°C incubated with 5% CO₂, and -20°C ([Figure 14](#)). Samples were stored in closed Eppendorf tubes additionally sealed with paraffin to minimize exposure to environmental moisture. Although relative humidity differed among the storage environments and was not specifically controlled, the additional sealing was intended to limit moisture exchange; therefore, temperature was considered the principal storage variable evaluated in this study. Particle concentration was measured using both NTA-PRO and VideoDrop, and particle retention (%) was calculated according to [Eq. 4](#) by normalizing the particle concentration measured at each storage time point to the corresponding Day 0 value.

Before filtration ([Figure 14.A](#)), NTA-PRO reported reduction in particle concentration over prolonged storage. Particle retention at 4°C progressively decreased from 91.0 ± 7.6% after 1 week to 60.9 ± 21.0% after 1 month and 27.5 ± 3.2% after 3 months. At RT, the corresponding values were 28.6 ± 28.3%, 44.6 ± 0.7%, and 24.6 ± 4.4%, while at 37°C they were 62.9 ± 5.6%, 36.9 ± 11.0 %, and 26.3 ± 0.6%. At -20°C, retention was 68.3 ± 27.9%, 43.9 ± 9.6%, and 45.1 ± 4.1%, respectively. VideoDrop similarly showed a progressive reduction in particle concentration with increasing storage duration. At 4°C, retention was 78.5 ± 24.9% after 1 week and 88.3 ± 3.0 % after 1 month and decreased to 35.7 ± 11.4% after 3 months. At RT, retention was 56.6 ± 7.6%, 61.6 ± 2.0%, and 33.7 ± 4.5%, respectively. At 37°C, particle concentration retention decreased from 57.1 ± 3.0% after 1 week to 22.3 ± 11.5% after 1 month and 34.6 ± 3.7% after 3 months, while at -20°C the corresponding values were 83.9 ± 21.8%, 76.5 ± 0.8%, and 53.5 ± 4.4%. Several statistically significant differences were observed between storage durations and conditions ($p < 0.05$), as indicated in [Figure14.A](#).

Following filtration ([Figure 14.B](#)), NTA-PRO showed a decline in particle concentration over time, with particle retention at 4°C decreasing from 80.5 ± 14.8% after 1 week to 60.9 ± 21.0% after 1 month and 38.7 ± 13.4% after 3 months. At RT, particle concentration retention was 81.0 ± 5.6%, 44.6 ± 0.8%, and 32.1 ± 4.1%, while at 37°C it decreased from 78.0 ± 9.9% to 36.9 ± 11.0% and

30.2 ± 1.5%, respectively. At -20°C, particle concentration retention was 87.00 ± 16.97%, 47.9 ± 3.1%, and 41.0 ± 10.18% after 1 week, 1 month, and 3 months, respectively. VideoDrop showed comparatively high particle concentration at 4°C and -20°C during the earlier storage periods. At 4°C, retention was 85.0 ± 19.8% after 1 week, 88.3 ± 3.0% after 1 month, and 65.5 ± 16.3% after 3 months. At RT, the corresponding values were 74.5 ± 16.3%, 66.1 ± 4.4%, and 44.9 ± 1.2%, while at 37°C retention was 76.0 ± 18.4%, 57.1 ± 3.0%, and 32.0 ± 28.4%. The -20°C condition showed 97.5 ± 0.7% after 1 week, 83.9 ± 21.8% after 1 month, and 85.1 ± 12.0% after 3 months. A statistically significant difference ($p < 0.05$) was observed between selected storage conditions following filtration.

Overall, the particle concentration data showed that retention generally decreased with prolonged storage, although the magnitude of particle loss depended on storage temperature, filtration status, and characterization technique. After three months, -20°C showed the highest particle concentration retention both before filtration and after filtration.

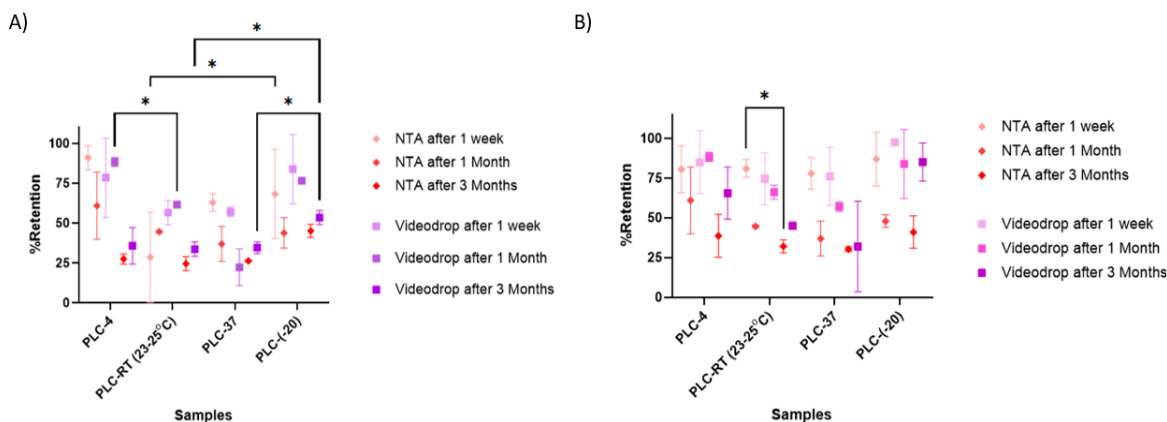


Figure 14. Comparison of particle concentration retention (%) of lyophilized placebo liposomes after three months of storage at 4°C, room temperature RT (23-25°C), 37°C incubate with 5% CO₂, and -20°C, as determined by NTA-PRO and VideoDrop. **(A)** Particle concentration retention before filtration and **(B)** particle concentration retention after filtration through a 0.22 µm membrane. Retention was calculated relative to the corresponding Day 0 particle concentration. Data are presented as mean ± SD. * $p < 0.05$.

4.6.2. Stability of GS liposomes

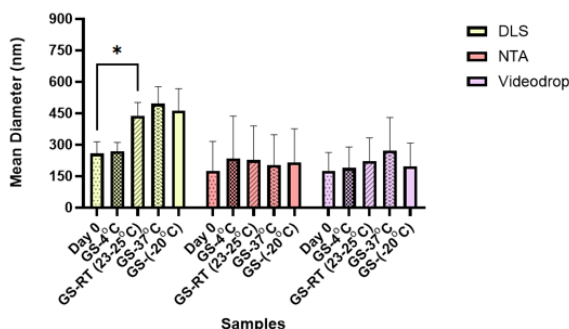
Particle size of GS-loaded liposomes was evaluated after one week of storage at 4°C, RT (23-25°C), 37°C (incubated with 5% CO₂), and -20°C, using DLS, NTA-PRO and VideoDrop before and after filtration through a 0.22 µm membrane ([Figure 15](#)). Samples were stored in closed Eppendorf

tubes additionally sealed with paraffin to minimize exposure to environmental moisture. Although relative humidity differed among the storage environments and was not specifically controlled, the additional sealing was intended to limit moisture exchange; therefore, temperature was considered the principal storage variable evaluated in this study.

Before filtration ([Figure 15.A](#)), DLS reported an increase in mean particle diameter from 258.2 ± 55.8 nm at Day 0 to 269.2 ± 41.5 nm at 4°C , 436.8 ± 65.4 nm at RT ($23\text{--}25^{\circ}\text{C}$), 495.1 ± 81.4 nm at 37°C , and 463.1 ± 104.1 nm at -20°C . The greatest mean diameter was recorded following storage at 37°C . Surprisingly, NTA-PRO measurements reported a similar range, with a Day 0 mean diameter of 174.0 ± 142.0 nm, compared with 235 ± 202 nm at 4°C , 227.0 ± 163.0 nm at RT, 204.0 ± 144.0 nm at 37°C , and 215.0 ± 161.0 nm at -20°C . VideoDrop measurements showed smaller changes in particle size, increasing from 176 ± 87 nm at Day 0 to 189 ± 100 nm at 4°C , 223 ± 110 nm at RT, and 272 ± 158 nm at 37°C , while the sample stored at -20°C showed a mean diameter of 197 ± 111 nm ([Figure 15.A](#)). Overall, the three instruments showed that storage influenced the particle-size distribution of GS liposomes, although the magnitude of the observed changes depended on the analytical technique. DLS showed the most pronounced increase in particle size, particularly at RT, 37°C , whereas NTA-PRO and VideoDrop generally reported smaller mean diameters.

Following filtration through a $0.22\text{ }\mu\text{m}$ membrane ([Figure 15.B](#)), the mean particle sizes were substantially reduced and showed greater agreement in the measurements campaign across the three instruments. In line with the filtration process, most of particle populations remained below 200 nm. This reduction was particularly evident for the conditions that exhibited large DLS diameters before filtration, suggesting the presence of larger particles or aggregates in the unfiltered formulations that were removed during filtration.

Mean Diameter of GS liposomes at after 1 week before filter



Mean Diameter of GS liposomes after 1 week after filter

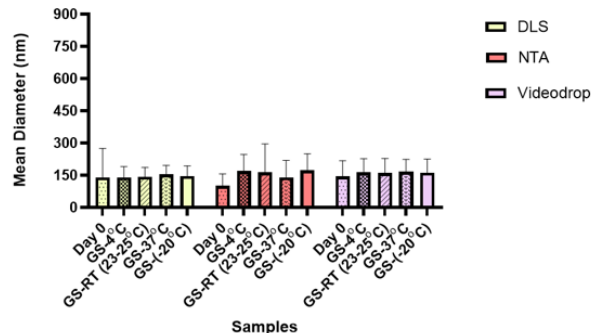
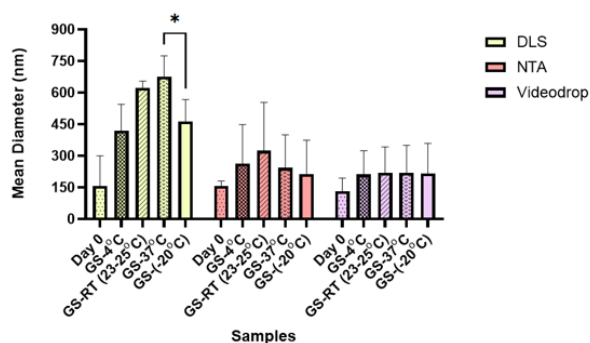


Figure 15. Mean particle diameter of gentamicin-loaded liposomes at Day 0 and after one week of storage at 4°C, room temperature RT (23-25°C), 37°C incubated with 5% CO₂, and -20°C, determined using DLS, NTA-PRO and VideoDrop: (A) before filtration and (B) after filtration through a 0.22 µm membrane. Data are presented as mean ± SD. *p < 0.05.

After one month of storage changes in the particle size of GS-loaded liposomes were measured. Before filtration ([Figure 16.A](#)), DLS showed a marked increase in mean particle diameter compared with Day 0 (258.2 ± 55.8 nm). The mean size increased to 419.2 ± 126.0 nm at 4°C, 621.9 ± 32.2 nm at RT, and 675.7 ± 99.5 nm at 37°C, while the sample stored at -20°C showed a mean diameter of 463.1 ± 104.1 nm. The largest DLS particle size was therefore observed at 37°C, which was significantly higher than the -20°C condition ($p < 0.05$), as indicated in [Figure 16. A](#). In comparison, NTA-PRO measurements showed greater variability, with the mean diameter increasing from 174.0 ± 142.0 nm at Day 0 to 263.0 ± 185.0 nm at 4°C and 324.0 ± 230.0 nm at RT, followed by 243.0 ± 157.0 nm at 37°C and 214.0 ± 161.0 nm at -20°C. VideoDrop reported substantially smaller and more consistent mean diameters of 176.0 ± 87 nm at Day 0, 212.0 ± 112 nm at 4°C, 220.0 ± 122.0 nm at RT, 219.0 ± 131.0 nm at 37°C, and 216.0 ± 144.0 nm at -20°C.

Following filtration ([Figure 16.B](#)), the mean particle sizes were considerably lower and showed greater agreement among the three analytical techniques. DLS measurements remained approximately 140–195 nm, while NTA-PRO measurements were approximately 100–145 nm across the investigated storage conditions. VideoDrop similarly showed mean particle sizes of approximately 140–180 nm. In contrast to the unfiltered samples, no significant differences among the storage conditions were observed following filtration.

Mean Diameter of GS liposomes after 1 month before filter



Mean Diameter of GS liposomes after 1 month after filter

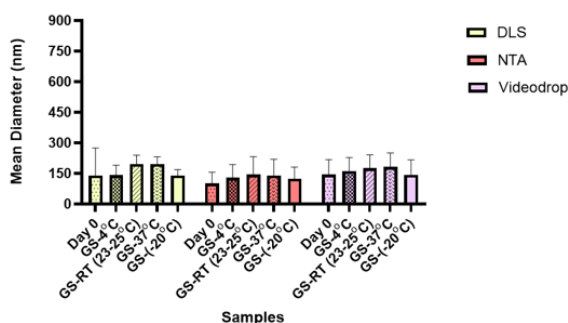


Figure 16. Mean particle diameter of gentamicin-loaded liposomes at Day 0 and after one month of storage at 4°C, room temperature RT (23–25°C), 37°C incubated with 5% CO₂, and –20°C, determined using DLS, NTA-PRO and Videodrop: (A) before filtration and (B) after filtration through a 0.22 µm membrane. Data are presented as mean ± SD. *p < 0.05

To proceed with the stability evaluation of the GS-liposomes, the evaluation of particle size was performed after three months of storage (Figure 17). Before filtration (Figure 17.A), DLS showed a pronounced increase in mean particle diameter compared with Day 0 (258.2 ± 55.8 nm), reaching 419.2 ± 126.0 nm at 4°C, 621.9 ± 32.2 nm at RT, 675.7 ± 99.5 nm at 37°C, and 463.1 ± 104.1 nm at –20°C. Several statistically significant differences were observed among the DLS storage conditions, with the largest particle sizes detected at RT and 37°C ($p < 0.05$ to $p < 0.0001$, according to the comparisons shown in Figure 17.A). In contrast, NTA-PRO measurements ranged from 174.0 ± 142.0 nm at Day 0 to 263.0 ± 185.0 nm at 4°C, 324.0 ± 230.0 nm at RT, 243.0 ± 157.0 nm at 37°C, and 214.0 ± 161.0 nm at –20°C. VideoDrop measurements remained relatively consistent across storage conditions, with mean diameters of 176.0 ± 87.0 nm at Day 0, 212.0 ± 112.0 nm at 4°C, 220.0 ± 122.0 nm at RT, 219.0 ± 131.0 nm at 37°C, and 216.0 ± 144.0 nm at –20°C.

Following filtration (Figure 17.B), the differences among the three characterization techniques were substantially reduced, with the particle populations remaining predominantly below 250 nm approximately. No statistically significant differences are accounted in the filtered samples. DLS showed considerably lower particle sizes after filtration than before filtration, while NTA-PRO measurements remained relatively consistent across the different storage conditions at approximately 100–140 nm. Similarly, VideoDrop reported mean particle diameters of

approximately 140–160 nm across the different storage temperatures. The reduction in DLS-measured particle size following filtration was particularly evident for samples stored at 4°C, RT, and 37°C, for which considerably larger mean diameters had been detected before filtration.

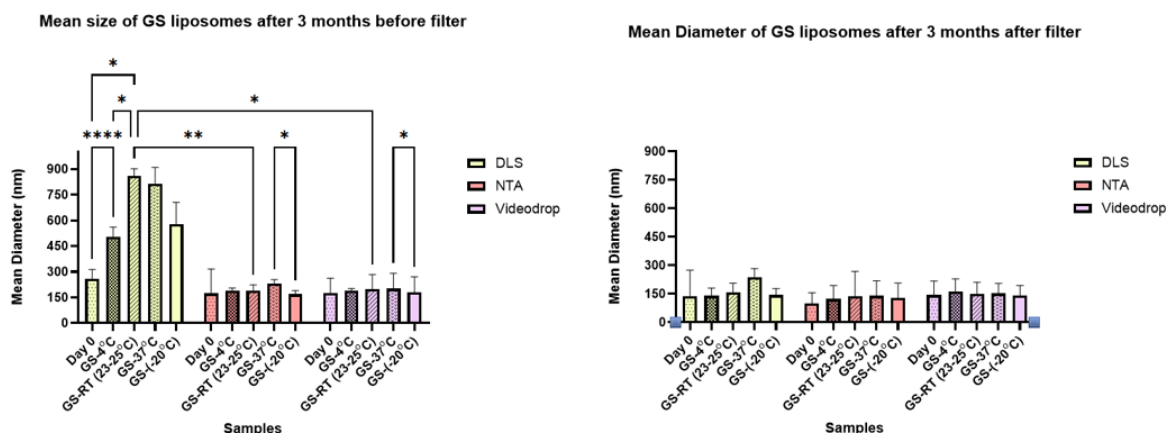


Figure 17. Mean particle diameter of gentamicin-loaded liposomes at Day 0 and after three months of storage at 4°C, room temperature RT (23–25°C), 37°C incubated with 5% CO₂, and –20°C, determined using DLS, NTA-PRO and Videodrop: (A) before filtration and (B) after filtration through a 0.22 µm membrane. Data are presented as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.0001.

Overall, after three months of storage, the largest changes in the unfiltered placebo and GS-liposome population were observed at RT and 37°C, while the samples stored at 4°C and –20°C showed a statistically non-significant increase in mean particle size relative to Day 0. Filtration substantially reduced the differences observed among the storage conditions and analytical techniques, indicating that a population of larger particles present in the unfiltered samples contributed to the increased mean diameters, particularly those measured by DLS. These results are reported in full in the supplementary data ([Supplementary Figures S13–S15](#)) for the placebo liposomes and ([supplementary data Figures S16–S18](#)) for the GS-liposomes.

4.6.2.1. Comparison of particle concentration retention of GS- liposome during storage

The stability of GS-loaded liposomes was further evaluated by comparing the percentage of particle concentration retained after 1 week, 1 month, and 3 months of storage at 4°C, RT (23–25°C), 37°C incubated with 5% CO₂, and –20°C using NTA-PRO and VideoDrop ([Figure 18](#)). Before filtration ([Figure 18.A](#)), particle retention generally decreased with increasing storage duration,

although the magnitude of the reduction depended on both storage temperature and analytical technique. The greatest retention was observed at 4°C and –20°C, whereas storage at RT and particularly 37°C resulted in greater particle loss. At 4°C, NTA-PRO showed a progressive reduction from approximately 30% after one week to below 20% after three months, while VideoDrop measurements remained approximately 45–50% across the investigated time points. At RT and 37°C, both techniques showed a statistically significant reduction in particle retention ($p < 0.05$ to $p < 0.0001$), with the lowest values observed after three months, particularly at 37°C (6–10% retention). At –20°C, VideoDrop showed comparatively higher retention throughout storage decreasing from approximately 42% to 28%, whereas NTA-PRO showed lower values and a gradual reduction over time. Several statistically significant differences were observed among storage conditions and time points, as indicated in [Figure 18.A](#).

Following filtration, the particle concentration retention was generally higher at the earlier storage time points compared with the corresponding unfiltered samples, ([Figure 18.B](#)). After one week, particularly high retention was observed at 4°C and –20°C, while a progressive reduction occurred with prolonged storage. By three months, NTA retention had decreased substantially across all temperatures. VideoDrop similarly showed reduced retention over time, although greater particle concentration retention was generally observed at 4°C and –20°C than at RT and 37°C. Significant differences among storage temperatures and storage durations were observed for both analytical techniques ($p < 0.05$).

Overall, the particle concentration data demonstrated a time and temperature-dependent reduction in GS-liposome retention, becoming more pronounced after three months of storage. Among the investigated conditions, 4°C and –20°C generally provided better preservation of the particle population, whereas 37°C showed the greatest particle loss, particularly after prolonged storage.

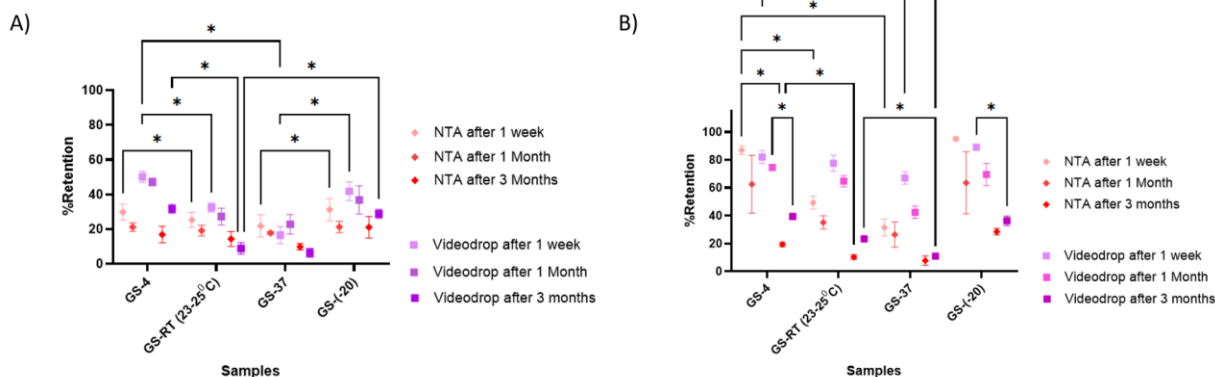


Figure 18. Particle concentration retention (%) of GS-loaded liposomes after 1 week, 1 month, and 3 months of storage at 4°C, RT (23-25°C), 37°C incubated with 5% CO₂, and -20°C, determined by NTA-PRO and Videodrop: (A) before filtration and (B) after filtration. Data are presented as mean $\pm$ SD. *p < 0.05

4.6.2.2. Gentamicin retention in the liposomes during storage

To further evaluate the degradation profile of GS-loaded liposomes, gentamicin retention within the liposomal formulation was assessed after 1 week, 1 month, and 3 months of storage at 4°C, room temperature (RT), 37°C, and -20°C ([Figure 19](#)). Gentamicin retention decreased with increasing storage duration under all investigated conditions. After one month, retention remained high at 4°C ($86.7 \pm 3.5\%$) and -20°C ($89.3 \pm 1.6\%$), whereas it decreased to $71.3 \pm 4.0\%$ at RT and $68.0 \pm 2.5\%$ at 37°C. At RT, the decrease from one week to one month was statistically significant ($p < 0.05$), while at 37°C the corresponding decrease was more pronounced ($p < 0.01$). After three months, gentamicin retention further decreased to $67.9 \pm 12.7\%$ at 4°C, $61.2 \pm 6.4\%$ at RT, $56.7 \pm 3.2\%$ at 37°C, and $62.9 \pm 6.4\%$ at -20°C. Compared with one week, the reduction after three months was statistically significant at 4°C ($p < 0.05$), RT ($p < 0.01$), 37°C ($p < 0.01$), and -20°C ($p < 0.01$). Overall, these results demonstrated a time- and temperature-dependent reduction in gentamicin retention, with 4°C and -20°C providing greater preservation during the first month, while prolonged storage for three months resulted in substantial drug loss under all conditions especially at RT and 37°C.

% Retention of Gentamicin after 3 months of storage after filter

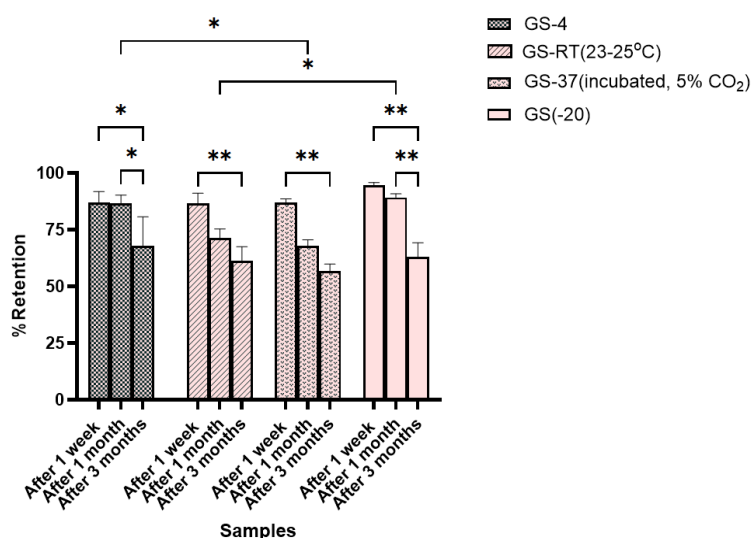


Figure 19. Gentamicin retention (%) in GS-loaded liposomes after 1 week, 1 month, and 3 months of storage at 4°C, room temperature RT (23-25°C), 37°C incubated with 5% CO₂, and -20°C. Data are presented as mean ± SD. *p < 0.05 and p** < 0.01.

5. Discussion

The present study tested the hypothesis that lyophilized gentamicin-loaded liposomes could retain their physicochemical properties and drug encapsulation after reconstitution while remaining responsive to electroporation-mediated drug release for therapeutic applications. The study further evaluated whether drug release was influenced by formulation composition and release environment, and whether the lyophilized formulation maintained its physicochemical properties and gentamicin content during shipment and shelf-life storage.

To address this hypothesis, the study focused on four main objectives, assessment of the effect of lyophilization and reconstitution on particle characteristics and gentamicin encapsulation, selection of the GS-loaded liposomal formulation based on its *in vitro* release behavior; evaluation of electroporation-triggered gentamicin release under different voltages, pH values, and release media; and assessment of the storage stability of the selected lyophilized formulation at different temperatures (4°C, room temperature RT (23-25°C), 37°C incubated with 5% CO₂, and -20°C).

The first objective was to determine whether the physicochemical characteristics and gentamicin encapsulation were preserved following lyophilization and reconstitution. The findings indicate that lyophilization allowed recovery of the 5:1 liposomal formulation with limited changes in particle characteristics and gentamicin encapsulation. Although particle size increased slightly following reconstitution, the difference was not statistically significant. Such changes could be associated with stresses occurring during freezing, dehydration, and rehydration, which can promote vesicle aggregation or fusion as supported by the existing literature [17]. However, these mechanisms were not directly investigated in the present study and therefore represent possible explanations rather than demonstrated causes. Importantly, encapsulation efficiency decreased only slightly, from before to after reconstitution. This limited change suggests that most of the initially encapsulated gentamicin was retained during freeze-drying and reconstitution. Importantly, encapsulation efficiency decreased only slightly following lyophilization and reconstitution, indicating that most of the initially encapsulated gentamicin was retained. This observation is consistent with the established protective role of trehalose during liposome freeze-drying presented in the literature [17].

The second objective was to select the most suitable GS-loaded formulation for subsequent *in vitro* and storage stability studies based on its release behavior. Comparison of the 3:1 and 5:1 formulation showed differences mainly during the early phase of release, while the profiles became less distinct over longer periods. The 5:1 formulation exhibited greater early gentamicin release, particularly following electroporation, and was therefore selected for the subsequent *in vitro* extended release, and stability investigations. These results demonstrate that formulation composition influenced the release response.

Shipment of the lyophilized formulations from Pavia to Dublin resulted in a negligible level of changes in mean particle diameters. This provides practical evidence that the formulations maintained their particle-size characteristics during shipment under the conditions used in this study. Comparison between DLS, NTA-PRO and VideoDrop instruments, demonstrated that measured particle size partly depended on the analytical techniques. Before filtration, DLS frequently reported larger mean diameters than NTA and VideoDrop, whereas agreement among the techniques generally improved after filtration. This observation is consistent with the greater

sensitivity of intensity-weighted DLS measurements to larger particles or aggregates, which can disproportionately influence the scattering signal [19]. In contrast, NTA-PRO and VideoDrop consistently reported smaller particle sizes, reflecting their particle-by-particle analytical approaches. The closer agreement among the three techniques following 0.22 μm filtration supports the presence of a fraction of larger particles or aggregates in the unfiltered samples [18]. Accordingly, these results highlight the value of using complementary analytical techniques rather than relying on DLS alone, particularly for the characterization of heterogeneous liposomal samples, in agreement with previous literature [19, 20]. In addition, NTA-PRO and VideoDrop provided particle-concentration measurements, which were particularly useful for monitoring changes during storage.

The fourth objective was to determine whether the reconstituted GS-loaded liposomes remained responsive to electroporation and whether the release response was affected by the experimental environment. Electroporation generally promoted earlier gentamicin release compared with the non-electroporated condition, demonstrating that the reconstituted liposomes retained responsiveness to electrical stimulation. This behavior is consistent with previous reports showing that electrical pulses can transiently increase lipid-bilayer permeability [21], thereby facilitating the release of hydrophilic gentamicin from the aqueous liposomal compartment. Despite the increased drug release, changes in the predominant nanoscale particle population measured by NTA-PRO and VideoDrop remained relatively limited. The larger diameters detected by DLS at some voltages may reflect a smaller population of larger particles or aggregates that disproportionately influenced the DLS signal. This further emphasizes the need for orthogonal characterization.

The release response was also dependent on the composition of the surrounding medium. More pronounced early release was generally observed under acidic conditions, particularly at pH 5.5, while PBS, saline, and HSA-containing culture medium produced distinct release profiles. These findings indicate that electroporation was not the only determinant of gentamicin release and that environmental conditions contributed to the observed release behavior. This is consistent with literature showing that pH, ionic strength, and proteins can affect lipid organization, electrostatic interactions, membrane permeability, and drug–membrane interactions [21,22].

The results therefore support the importance of evaluating electrically triggered liposomal release under physiologically relevant media rather than under a single buffer condition.

The fifth objective was to assess the stability of the lyophilized formulation during storage under different temperature conditions 4°C, room temperature RT (23-25°C), 37°C incubated with 5% CO₂, and -20°C). Particle size range, particle concentration, and gentamicin retention changed with both storage duration and temperature. Among the investigated conditions, storage at 4°C generally provided better preservation of particle concentration and gentamicin retention, whereas greater deterioration was observed at elevated temperature, particularly at 37°C. Elevated temperatures can accelerate lipid oxidation and hydrolysis and promote membrane rearrangement and aggregation, providing possible mechanisms for the observed changes [8]. Overall, the combined particle-concentration and gentamicin-retention (%) results support 4°C as the more suitable of the investigated storage conditions over the evaluated period, consistent with previous reports on the temperature-dependent stability of liposomal formulations [23].

An additional factor that should be considered when interpreting the stability findings is environmental humidity. The lyophilized samples were stored in closed Eppendorf tubes additionally sealed with paraffin to minimize moisture exchange. However, relative humidity differed among the storage environments and was not experimentally controlled. Therefore, although temperature was the principal storage variable investigated, a contribution from residual moisture or moisture ingress cannot be completely excluded, since moisture can affect the physical stability of lyophilized formulations.

Taken together, the findings address the central hypothesis by demonstrating that the selected GS-loaded liposomal formulation could be lyophilized and reconstituted with limited changes in particle characteristics and gentamicin encapsulation and remained responsive to electroporation. Furthermore, while drug release depended on formulation and environmental conditions, the physicochemical and drug stability progressively changed during storage, highlighting the need for regular characterization as quality control and shelf-life assurance.

Several limitations should be considered. The nature of the larger structures detected in some samples was not directly identified, and therefore aggregation or fusion cannot be conclusively

established. The release experiments were performed *in vitro* and cannot establish the behavior of the formulation *in vivo*. Furthermore, the evaluated stability period is insufficient to establish a definitive pharmaceutical shelf-life, and longer-term measurements, including the planned six-month time point, are required. Relative humidity and residual moisture were not directly controlled or quantified during storage, and the homogeneity of aliquots collected from individual lyophilized batches was not specifically evaluated. Future studies should therefore include longer-term stability testing, controlled humidity and residual-moisture measurements, assessment of batch and aliquot homogeneity, and evaluation in more complex biological models.

6. Conclusion

Overall, this work provides a foundation for the development of a stable, lyophilized and electrically responsive liposomal drug-delivery platform. The ability to preserve the formulation during shipment and shelf-life storage and then to subsequently modulate drug release through an externally applied electrical stimulus was presented in details. This represents a promising platform solution for achieving greater spatial and temporal control over drug delivery. In addition, storage at 4°C resulted in the most favorable preservation temperature; whereas both 4°C and –20°C promoted gentamicin retention. Future work should next complete the 6-months stability study. Complementarily, a comparative study with liposomal suspensions, as freshly produced under the same microfluids' parameters as investigated in this study, should be carried out. Going forward for this study, further biological evaluation under physiologically or *in vitro* cellular models establish the therapeutic potential of this lyophilized liposomal platform, as electroporation-responsive delivery system.

8. References

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The Supplementary Data

https://drive.google.com/file/d/1EnUa_KjzJSF5QUZba2vbbiQRYwh_iqkw/view?usp=sharing