Journal of Physical Chemistry & Biophysics

OPEN ACCESS Freely available online

Research Article

Impact of Type of Nebulizers on Liposomes Prepared by Bed Side Reconstitution Technique

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ABSTRACT

Bedside reconstitution techniques for liposome preparation at the time of drug administration eliminate the stability issue of liquid liposomes products. In this study of Tobramycin sulfate liposomal formulation has been developed as an inhaled therapy for Pseudomonas aeruginosa infection in the lungs. To analyze the effectiveness of Pre and Post drug delivery of liposomal drug formulation, this research article utilizes three types of Nebulizers—Ultrasonic Nebulizers, Mesh Nebulizer, and Compressed Nebulizer. Post nebulization, physicochemical characteristics, and stability of liposomes were evaluated and compared with pre-nebulization characteristics of the liposomes. Ultrasonic nebulization provided better product attributes compared to Mesh and compressed nebulizer based on the analysis performed using Transmission Electron Microscopy, HPLC, Particle size, Zeta Potential, and TCS-PC. The main goal was to determine the entrapment efficacy of the drug in liposomes as well as to determine the liposomal stability and size especially after using the nebulizer as a drug delivery tool to ensure the amount of drug delivered. The statistical data has shown that there is no significant 68 difference in Particle size and entrapment efficacy pre- and post-nebulization using various nebulizers.

Keywords: Liposomes; Compressed nebulizer; Drug delivery; Nanotechnology; Mesh nebulizer; Ultrasonic nebulizer

INTRODUCTION

The growing rate of pulmonary infection has led to the development of various methods for cure and treatment. The basic measurements that are being taken are by using the different mechanisms or routes of drug delivery to increase the lung deposition of the drug. One of the major technologies being used is nano-drug delivery as liposomes. Liposomes that were initially used as a biological model are now being used as an alternative for faster drug delivery due to their property to alter pharmacokinetics/bio distribution and to attenuate toxicity. This research paper mainly focuses on Liposomal aminoglycoside drugs that are majorly used for respiratory infection-their formulation, stability, size, and entrapment efficacy pre, and post-nebulization. Since Liposomes have better penetration through the epithelial lining and the aminoglycoside drugs are used as an upper respiratory lung infection with hydrophilic nature, so with the combined effect of entrapped drug within the liposomes, it's easier to fight pulmonary infection by targeting the specific area as well as by increasing the loaded drug to counter resistance development. We have designed a liposomal formulation of the antibiotic Tobramycin Sulfate [1,2] for the inhalation treatment of cystic fibrosis caused due to Pseudomonas aeruginosa infection [3,4]. In this research paper, we performed an *in vitro* analysis of liposomes by using both pre and post nebulization with three types of nebulizers—Ultrasonic, Mesh, and Compressed to show their effect on size, shape, and entrapment efficacy. Nebulization is the easiest method to deliver liposomes in the lungs but the applied stress with various nebulizers can affect the liposome formulation efficacy as well as properties of liposomal drugs. Keeping all the criteria in place this research will try to demonstrate whether nebulized liposomes vary as a function of droplet size, as droplet size impacts lung deposition. Distinct properties liposomes could be deposited in different areas of the lung that is central *vs* peripheral part. The best method that is utilized for the analysis of the liposomes is *via* TEM/ Particle sizing that can help with the size and structure of liposomes

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Received: 04-May-2022, Manuscript No. JPCHS-22-17717; **Editor assigned:** 09- May -2022, PreQC No. JPCHS-22-17717 (PQ); **Reviewed:** 23- May -2022, QC No. JPCHS-22-17717; **Revised:** 29-May-2022, Manuscript No. JPCHS-22-17717 (R); **Published:** 06-Jun-2022, DOI: 10.35248/2161-0983.22.9.256

Citation: Aggarwal P, Patel V, Sarvaiya J (2022) Impact of Type of Nebulizers on Liposomes Prepared by Bed Side Reconstitution Technique. J Pharma Care Health Sys.9:256.

and HPLC that can help with the analysis entrapment efficacy of the formulation. Characterization methods that are further used in this study are Lifetime decay using TCS-PC to predict decaying of each sample and average lifetime [5]. Formulation of liposome are adjusted after several trails to maintain the lipid to drug ratio alongside the phospholipid's combination. The commonly used phospholipids in preparation of liposomes are: Phosphatidylcholine (PC), Phosphatidylserine (PS), Phosph -atidylethanolamine (PE), Phosphatidylinositol (PI), 1,2-dioleoyl -sn-glycero-3-phospho-L-serine, Distearoylphosphatidylcholine (DSPC), Dipalmitoylphosphatidylserine, Dipalmitoylphosphat idylcholine (DPPC), dipalmitoylphosphatidylglycerol, Dioleoyl phosphatidylethanolamine (DOPE).

MATERIALS AND METHODS

Materials Tobramycin Sulphate, Cholesterol (CHL), DPPC and S-100 were obtained from Sigma Co. Acetonitrile and methanol obtained was of High-Performance Liquid Chromatography (HPLC) grade from Sigma Co. Column: Syncronis C 18 HPLC column (Thermo fisher scientific). Various generic nebulizers based on different principle were used.

Liposome preparation method

Liposomes samples were prepared by a conventional injection method containing tobramycin sulfate with phospholipids in the ratio of S-100, Cholesterol, and Dipalmitoylphosphatidylcholine (DPPC) in 3:1:2 mixed with 1 ml ethyl alcohol. The concentration of Tobramycin Sulphate 0.05 mg per mg in an aqueous solution was combined with total lipid. The liposome preparation method uses different. 1 ml of 0.9%Nacl was mixed with 300 mg of aminoglycoside drug. Through injection method, 1 ml ethyl phospholipid preparation was injected using 21 G needle in 1 ml tobramycin Nacl solution to make liposome formulation and further dilution was made up to 10 ml by adding 0.9%Nacl. 2 ml of liposomes were filtered through centrifugation tube 20K Dalton size and centrifugation was performed at 5000 g 177 at 15°C–20°C for 15 min. Liposomes prepared using the mentioned method usually range between mean 178 diameters of 200–500 nm further manual shaking is done to control the size distribution. Before analyzing any formulation as above unencapsulated tobramycin sulfate and the residual solvent were removed using a diafiltration assembly with 0.9% NaCl solution. The tobramycin concentration was measured as 300 mg/mL before use. Liposome diameter was measured by quasi-elastic light scattering using particle Sizer (Malvern Instruments). Before the nebulization, the lipid to drug weight ratio, average liposome diameter, and percentage of total drug that is linked with the liposomes that are 184 observed in the study was found to be 0.68, 470.22 nm, and 95%, respectively [6].

Method of nebulization

Nebulizers are mainly used for inhalation therapy by the population that cannot use Metered dose inhalation [7-9]. Also, nebulization is effective to convert Liposomal liquid drug formulation to mist without much disruption of structure by using mechanical and thermal energy [10-13]. Three most advance type of non-invasive mechanical and commercially available nebulizers are

• Mesh Nebulizer which is based on venturi principle and uses low frequency waves are effective as they use micropump technology, to force liquid medications through multiple apertures mesh. They are quit and portable with less than 10% residual volume. The best suited particle sizes up to 5 µm. Collection procedure follows placing compressed nebulizer 15 cm from the collecting chamber connected through corrugated tube, sample was collected by condensation process.

• Ultrasonic Nebulizer is based on a piezoelectric crystal vibration (electrical energy to extremely rapid mechanical vibration). Ultrasonic nebulizer with size ranging from 1-5 micrometer with residual drug volume up to 30% [14,15]. Collection procedure follows the Ultrasonic nebulizer placed 15 cm from the collecting chamber connected through corrugated tube, ultrasonic nebulizer connected with other chamber attached through another corrugated tube and the chamber were cooled using icepack for sample collection [16].

• Compressed Nebulizer are based on the principle of pressurized air passes through the various tubes on the medical nebulizer compressor, it quickly turns the medicine into smaller mist particle with particle size ranging from 2-5 micrometer with residual drug volume up to 40%. This nebulizer is bulky as compared to other two nebulizer. The sample is collected using corrugated tube and sample were condensed and collected and analyzed. Aerosolization of each liposomal Tobramycin sulfate was performed, using 4 mL of liposomal formulation with concentration as 30 mg/ml and was placed in above-mentioned nebulizer liposomes was collected post nebulization and characterization analysis was performed as below in Figure 1.

Determination of the amount of tobramycin sulphate entrapped in the liposomes

HPLC assay for the determination of tobramycin sulphate: Qualitative and quantitative analyses of aminoglycoside drugs in liposome formulations were performed by using an HPLC [17]. Chromatographic Conditions for HPLC were as follow, Mode: LC, Detector. High-performance liquid chromatography was performed isocratically at ambient temperature and a flow rate of 1.2 mL/min with Ultra Violet (UV) detection at UV 365 nm, Injection volume: 20 µL. The mobile phase mixture is as follows: 2.0 gm of Tris aminomethane was dissolved in about 800 ml of water. 20 ml of 0.1 N Sulfuric acid was added and the entire solution was diluted with acetonitrile up to 2 L. The sample was further cooled and filtered through a 0.2 micron pore size filter. The retention time of the Aminoglycoside drug was 9.9 min. Every sample was filtered through a 0.22-micron membrane filter, prior to injection onto the HPLC column [18,19]. Before performing the Entrapment efficacy study Linearity of HPLC was determined from five working standard solutions of aminoglycoside drug in Acetonitrile. Over the concentration range as 25 mg, 50 mg, 75 mg, 100 mg, 125 mg, 150 mg, 175 mg, 200 mg. The correlation (R^2), intercept, and slope of standard curves was calculated. The R square value was found to be 0.99. The peak areas of all test samples were calculated and compared with the standard curve peak, to determine the amount of aminoglycoside drug [20]. These experimental trials were conducted multiple times to assure the result. Post Linearity of the drug, entrapment efficacy of liposomes was calculated using HPLC pre nebulization and post nebulization using mesh and ultrasonic nebulizer. As per drug peak area, the Ultrasonic nebulizer shows better entrapment efficacy as compared to Mesh Nebulizer and compressed nebulizer (Figures 2-5) (Graph 1) [21].

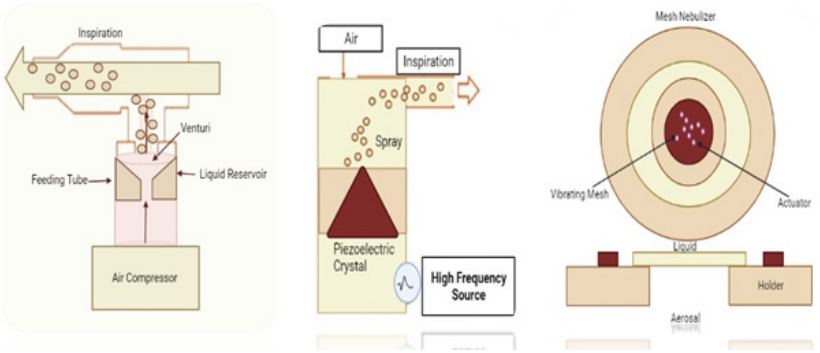


Figure 1: Representation of principle of compressed nebulizer (a), Ultrasonic Nebulizer(b) and Mesh Nebulizer(c).

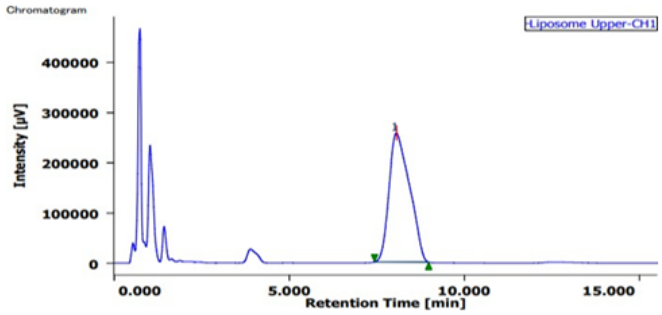


Figure 2: Entrapped area of pre-nebulizer liposomes.

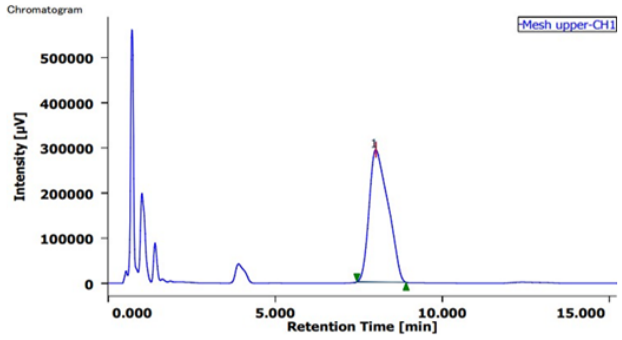


Figure 3: Entrapped area of mesh nebulizer liposomes.

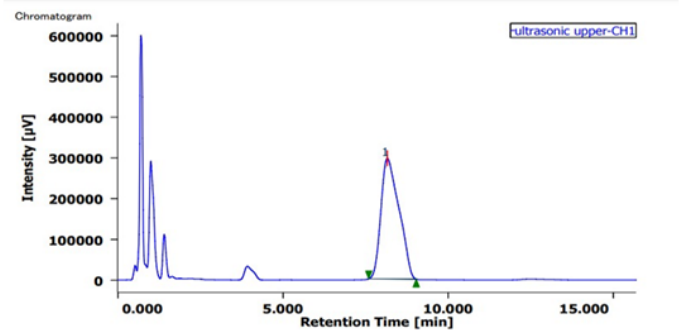


Figure 4: Entrapped area of ultrasonic nebulizer liposomes.

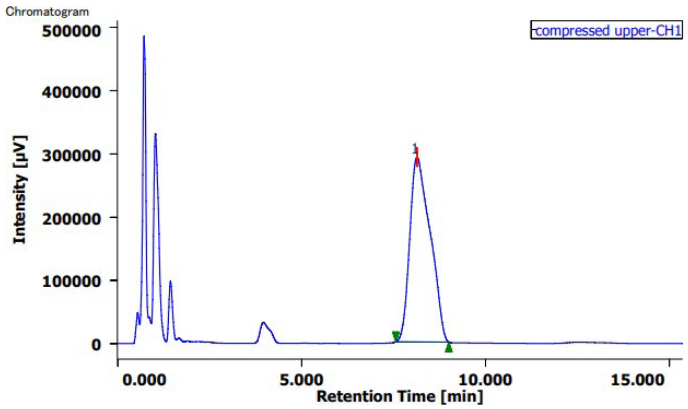
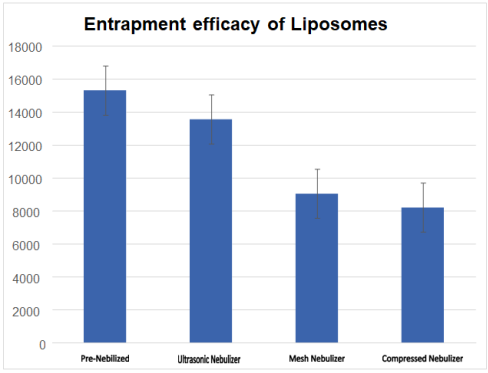


Figure 5: Entrapped area of compressed nebulizer liposomes.



Graph 1: Graph representing entrapment efficacy of liposomes pre and post nebulization.

Advances in micro-structure characterization of liposomes pre and post nebulization

Characterization of liposomes based on size (Particle sizing): The size of the Liposome as well as the polydispersity index aka PDI was measured using a Nanosizer ZS (Malvern Instruments) version 2.2 *via* the process of Dynamic Light Scattering (DLS) (Graph 2). The liposomes formulation was diluted to 1/3rd and was analyzed using zeta cuvette and Z average was taken [22,23]. The same procedure was taken to predict the average Z value 358.5 and PDI below 0.500 (Figure 6).

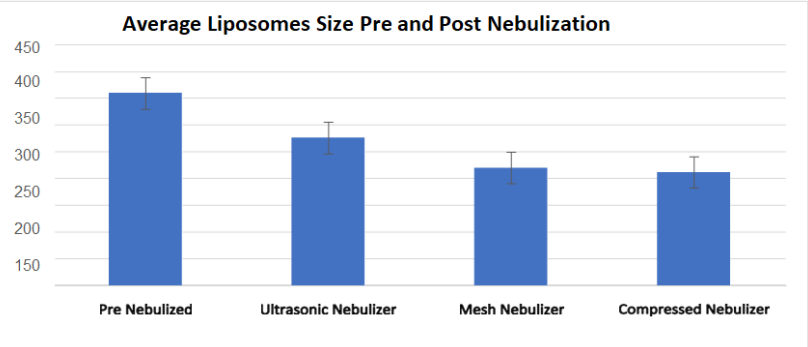
Transmission Electron Microscope (TEM)

Transmission Electron Microscope model Tecnai 20, Make: Philips, Holland was used for analysis with accelerating Voltage 200 kv, Electron Source as W emitter and LaB6 and magnification as 25X to 750000X or higher. For the fundamental understanding of a liposomal tobramycin sulfate formulation with a thorough characterization of the material morphology, crystal structure, interface structure, surfaces and defects all have their influence on the properties of material. Lamellarity of liposomes was determined using transmission electron microscopy for liposomal formulation as a powerful technique for studying a range of liposomal size at nm level 269 [24,25]. Liposome formulation were freshly prepared using injection method as mentioned above and were analyzed using TEM analysis as shown in Figures 7-10 below. Liposomes were nebulized using Ultrasonic, Mesh and Compressed nebulizer. Pre and post nebulized samples were collected and analyzed using TEM (Graph 3).

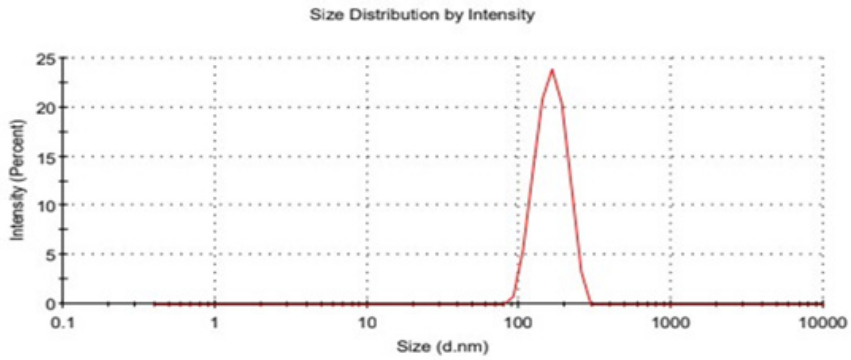
As shown in above TEM images, not much disruption/difference in size is seen by using all three nebulizers except for slight non-uniformity that is seen under Figure 4. Liposomes size ranges from 100 nm to 500 nm are seen in all analyzed samples Table 1.

Characterization based on life time decay

Time-resolved fluorescence measurements: Time-correlated single-photon counting aka TCS-PC is a cutting-edge method to measure fluorescence decays in the time domain. The principle behind TCS-PC is single-photon events that are detected and their time of arrival which is correlated with the laser pulse, which is used for sample excitation with the high repetition rate [26]. The sample additionally emits the photon that is detected with a high gain photon multiplier and time with respect to excitation pulse is measured and *via* counting these events the photon distribution histogram over rime is built up. With the advanced DeltaFlex TCS-PC system, the measurement is done for luminescence lifetime of the sample ranging over 11 orders of magnitude and measure of lifetimes from 25 ps to 1 second. The data are given in Table 1 shows the Lifetime decay for both pre and post nebulization of liposomes using mesh, ultrasonic nebulizer, and compressed nebulizer shows the average lifetime from 0.7 ns–1.1 ns that remains similar for all four samples as well the overlay graph shows the sameness in the formulations (Tables 2 and 3) (Graph 4) [27].



Graph 2: Average liposomal formulation with Z value 358.5nm and PDI value at 0.471.



Results				
ZAverage(d.nm): 358.5	Peaks	Size (d.nm)	% Intensity	St Dev (d.nm)
Pdl: 0.471	Peak 1:	165.1	100	36.83
Intercept: 0.954	Peak 2:	0	0	0
Result quality: refer to quality report	peak 3:	0	0	0

Figure 6: Representation of average Liposome size.

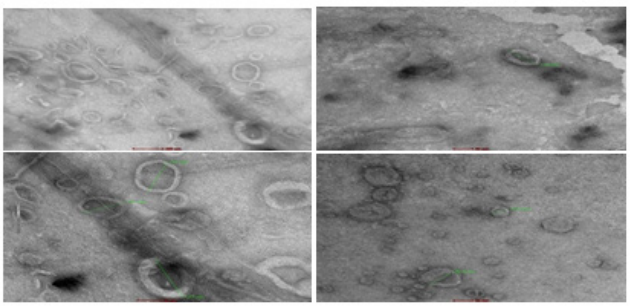


Figure 7: Representation of liposomal formulation pre-nebulization under the scale range from 20-100 nm.

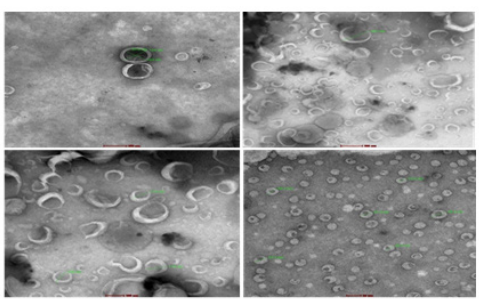


Figure 8: Representation of liposomal ultrasonic-nebulization under the scale range from 20-100 nm.

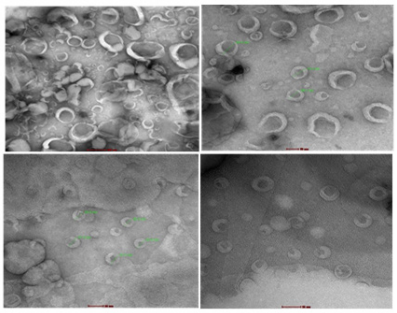


Figure 9: Representation of liposomal mesh-nebulization under the scale range from 20-100 nm.

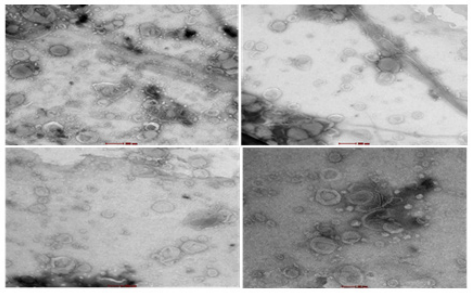
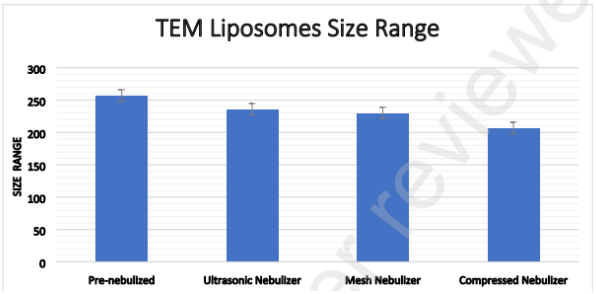


Figure 10: Representation of liposomal compressed-nebulization under the scale range from 20-100 nm.



Graph 3: Average liposomal formulation size range of pre and post liposomal formulation using various nebulizers.

Table 1: Data of statistical analysis and P value (<0.50) comparison using two tailed P value between pre and post-nebulization of liposomes.

Statistical analysis			
Liposomes comparison	Pre-nebulization and ultrasonic nebulization	Pre-nebulization and mesh nebulization	Pre-nebulization and compressed nebulization
Two-Tailed P value	0.4368	0.1315	0.081
Mean	16	30.33	40.33
95% confidence interval	-32.69 to 64.69	-12.95 to 73.62	-7.21 to 87.87
Significance	The difference is considered to be not statistically significant	The difference is considered to be not statistically significant	The difference is considered to be not statistically significant

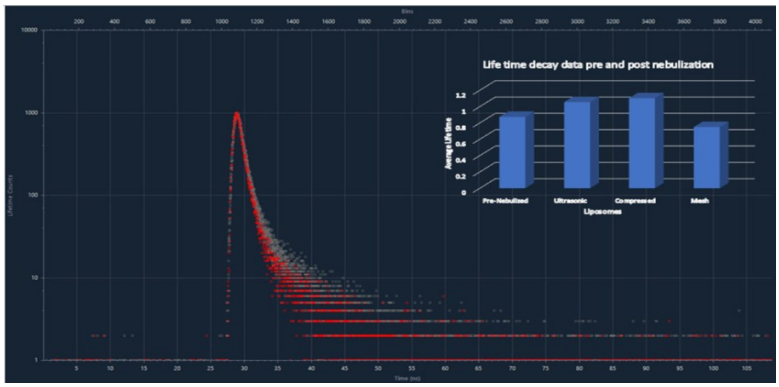
Table 2: Representation of Life time decays both pre and post nebulization with chi sq. value under 1.4 and wavelength/nm at 410.

Pre-nebulized liposomes	Ultrasonic nebulizer	Compressed nebulizer	Mesh nebulizer
1-5 exponentials	1-5 exponentials	1-5 exponentials	1-5 exponentials
Decay-1	Decay-2	Decay-3	Decay-4
Value	Value	Value	Value

T1	0.796017	T1	1.05503	T1	1.43435	T1	0.74861
T2	0.125336	T2	1.04299	T2	0.76329	T2	0.0745673
T3	3.36088	T3	3.21316	T3	6.6446	T3	2.65568
A	1.9708	A	1.75603	A	-0.586141	A	1.95202
Average lifetime/ ns`	0.87379678	Average lifetime/ ns	1.0549090	Average lifetime/ ns	1.10232334	Average lifetime/ ns	0.74916200
Chi sq.	1.1572878	Chi sq.	1.1604787	Chi sq.	1.1082720	Chi sq.	1.3046543

Table 3: Data of statistical analysis and P value comparision using two tailed P value between pre and post-nebulization of liposomes.

Statistical analysis			
Liposomes comparison	Pre-nebulization and ultrasonic nebulization	Pre-nebulization and mesh nebulization	Pre-nebulization and compressed nebulization
Two-Tailed P value	0.7851	0.803	0.7521
Mean	-0.199057844	-0.189358096	-0.446318512
95% confidence interval	1.8265588156-1.4284431276	1.5040853279-1.8828015199	3.5941399623-2.7015029385
Significance	The difference is considered to be not statistically significant.	The difference is considered to be not statistically significant.	The difference is considered to be not statistically significant.



Graph 4: Representation of Life time decay for Pre and Post nebulization of liposomal formulation using mesh, ultrasonic and compressed Nebulizer.

RESULTS AND DISCUSSION

Tobramycin sulfate inhalational liposomal formulation was developed for the treatment of Pseudomonas aeruginosa infections in cystic fibrosis. This study was designed to predict the stability of the formulation post nebulization after bedside reconstitution of the liposomes. Studies with liposomes and the various trail have shown that any stress applied to formulation impact the stability of the liposomes. Currently, trials are conducted to predict the effective dose and stable formulation. The dose-volume examined for this study is 4 ml (240 mg tobramycin sulfate), which was filled in various nebulizers utilized. The results from TEM have shown mostly unilamellar liposomes are present in the majority. Liposome particle size for Tobramycin liposomal formulation is smaller and falls in the size range of 2-5 µm. Based on the result obtained from liposomal formulation pre and post-nebulization and its analysis using various methods such as TEM, Zeta sizing and TCS-PC it can be concluded that there is not much effect seen on the formulation post nebulization of Liposomes in terms of size and efficacy. As per the above study post nebulization of liposomes, the size uniformity can be seen and the statistical data has suggested that there is no significant difference in pre and post-nebulization of the formulation. With the comparative study, the ultrasonic nebulization has shown better efficacy as compared to mesh and compressed nebulizer.

CONCLUSION

As liposomal inhalation has shown faster growth in therapy various question based on its stability is obvious to present the formulation in the market. With this current study, data has suggested that post nebulization the liposomes remains intact and the entrapment efficacy varies by a minor percentage. The bedside preparation of liposomes using our mentioned method of liposomal formulation and preparation has shown stability post nebulization. Hence further studies can be conducted on a similar basis.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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